Express Mail Label No. EV 292458974US
Date of Deposit: February 5, 2004
Docket No. C0989.70049US01

Figure 1

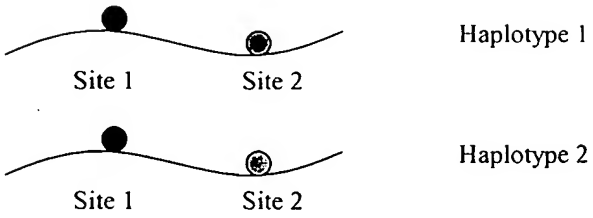


Figure 2

Figure 3

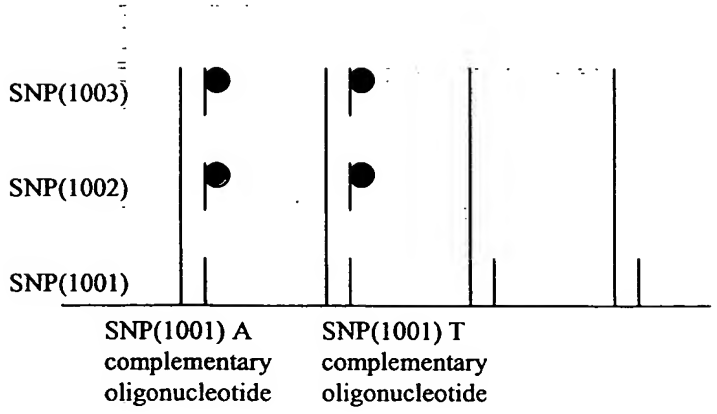


Figure 4

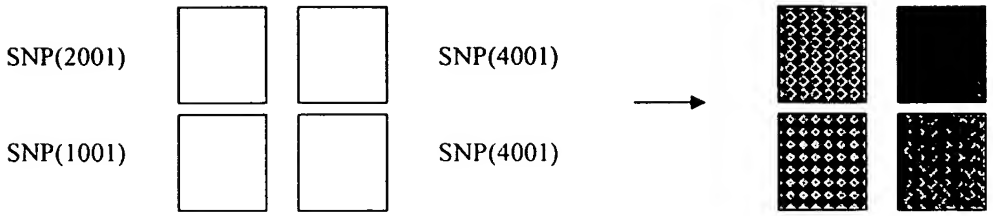


Figure 5

Figure 6

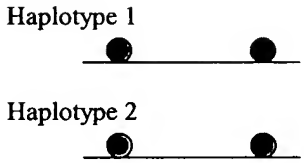


Figure 7

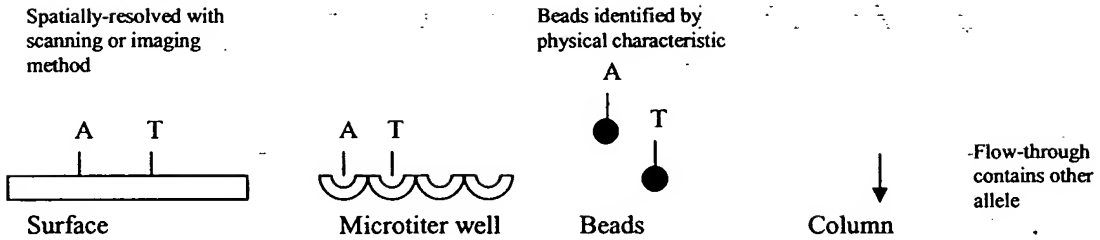


Figure 8

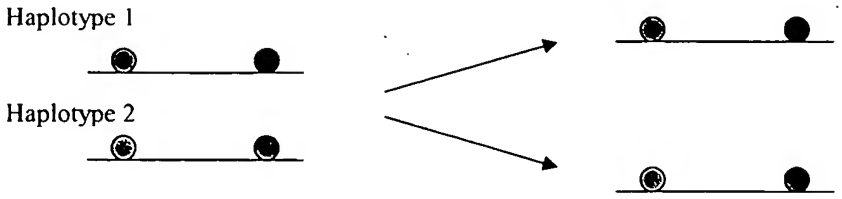


Figure 9

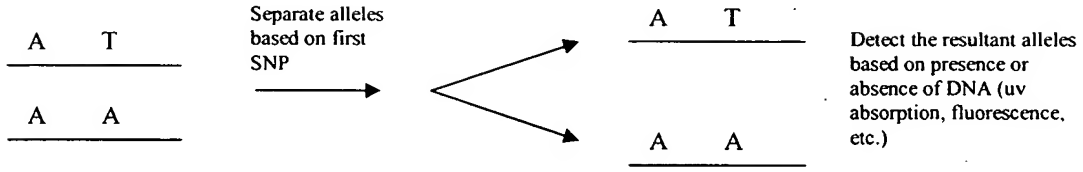


Figure 10

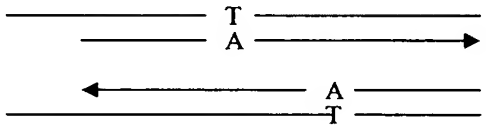
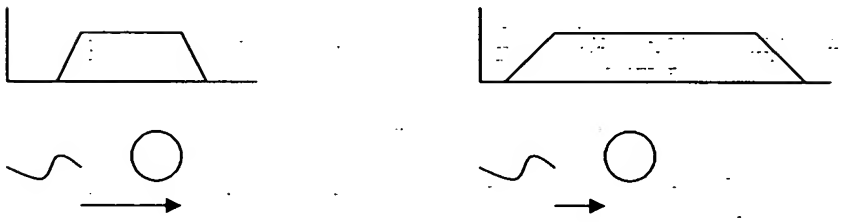


Figure 11



V = 500 microns/second

Integrated intensity = 1000 units (arbitrary)

V = 250 microns/second

Integrated intensity ~ 2000 units (arbitrary)

Figure 12

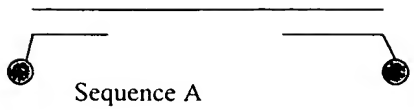
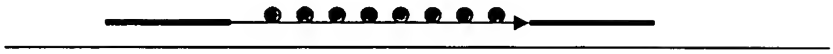


Figure 13



Fluorescent nucleotide incorporation
with polymerase incorporation.

Figure 14

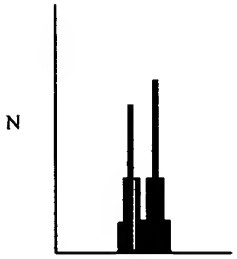


Figure 15

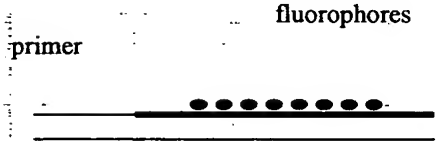
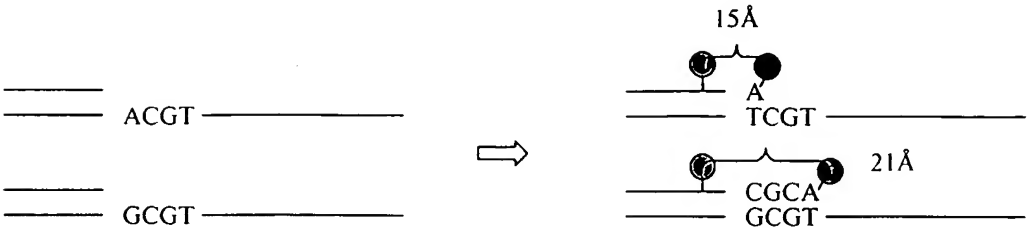


Figure 16



A/G SNP detected through primer extension-spFRET and differential termination using ddATP

Figure 17

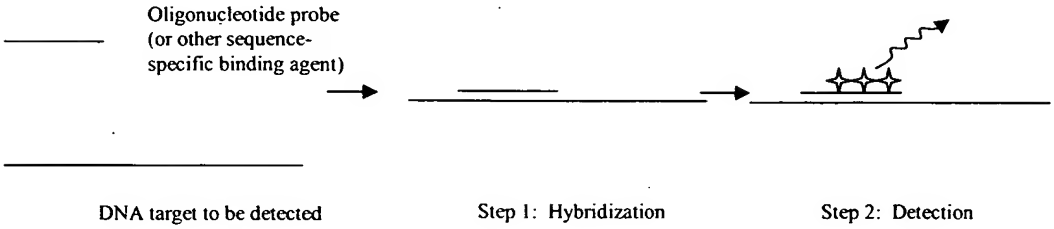


Figure 18

TWO-COLOR PRIMER EXTENSION ASSAY

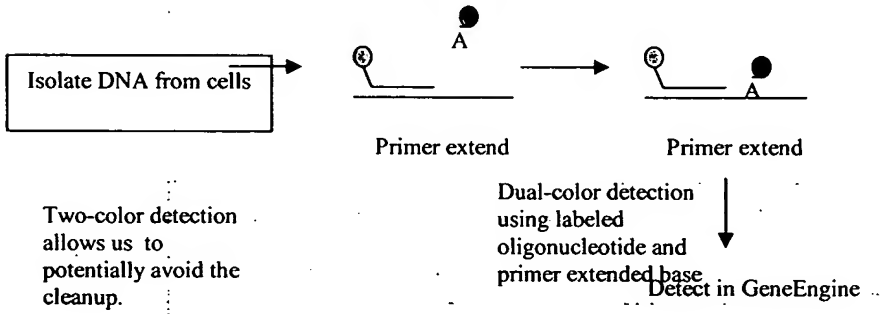


Figure 19

TWO-COLOR EXTENSION AND LIGATION ASSAY

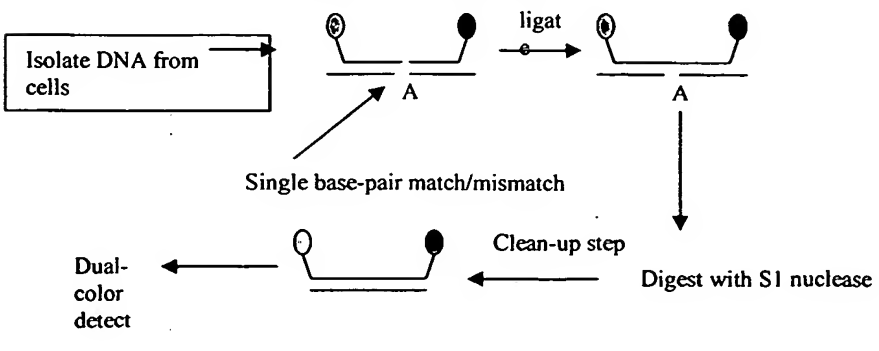


Figure 20

spFRET-BASED ASSAY OR PRIMER EXTENSION ASSAY-BASED CLEAVAGE OF PRODUCT (LIKE TAQMAN)

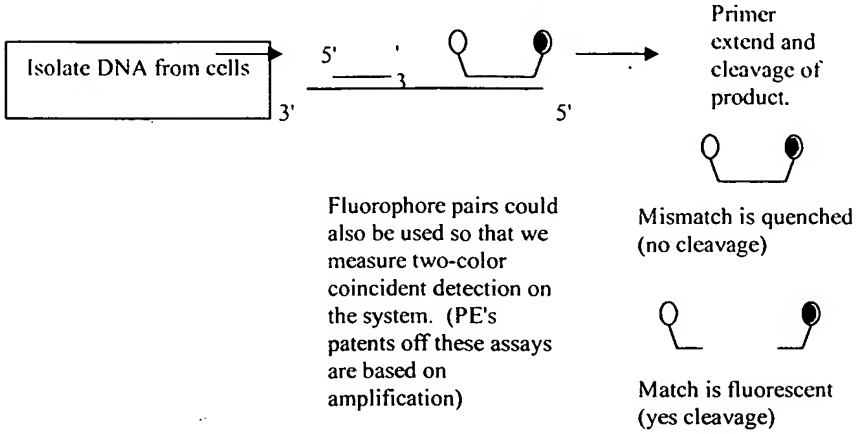


Figure 21

spFRET-BASED ASSAY BASED ON COINCIDENT HYBRIDIZATION

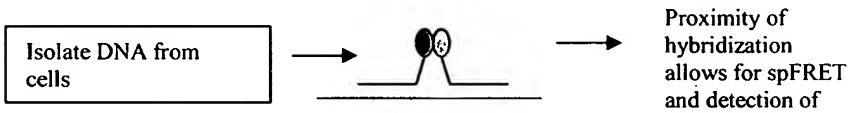


Figure 22

spFRET-BASED ASSAY WITH COMBINATION OF SINGLE-BASE EXTENSION REACTION

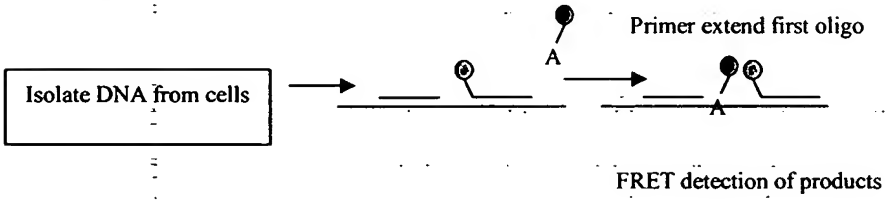


Figure 23

TWO-COLOR DETECTION ASSAY IN COMBINATION WITH PRIMER EXTENSION

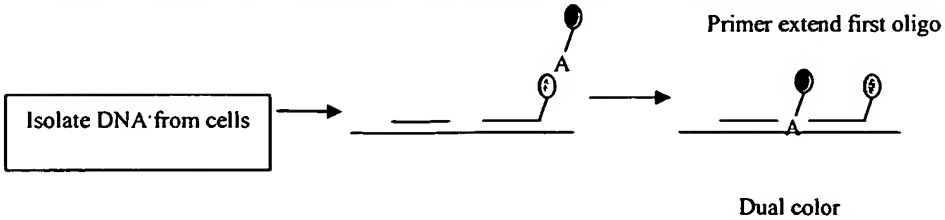


Figure 24

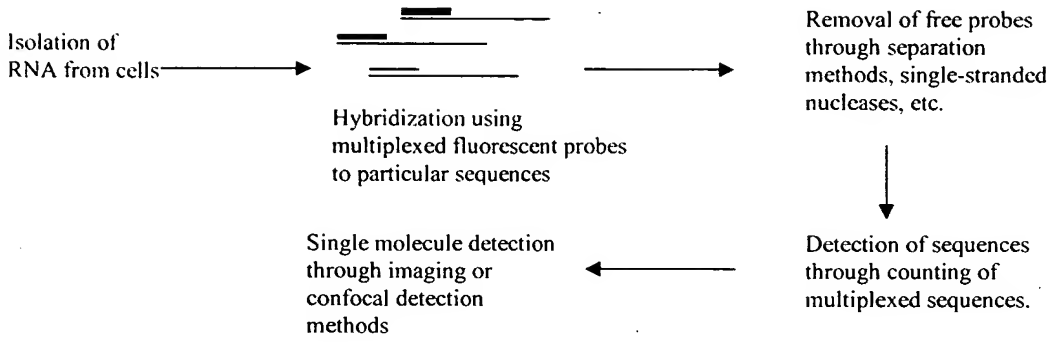


Figure 25

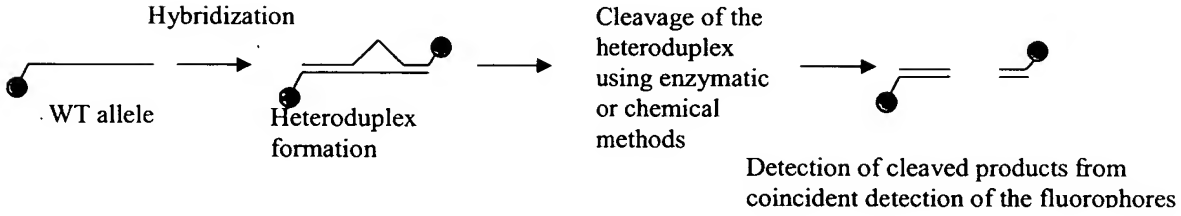


Figure 26

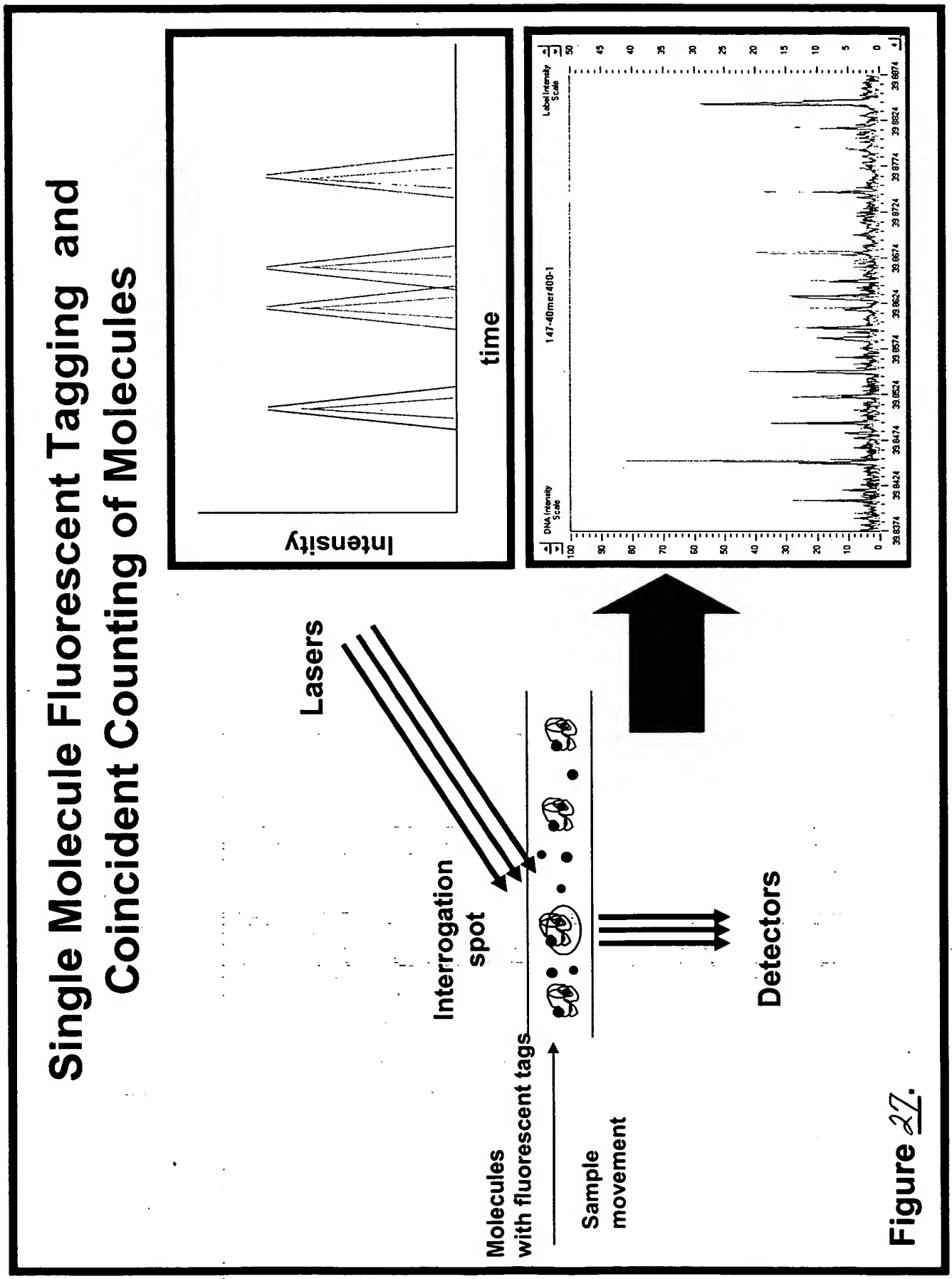

Single Molecule Fluorescent Tagging and Coincident Counting of Molecules
Intensity
time
Lasers
Interrogation spot
Molecules with fluorescent tags
Sample movement
Detectors

Figure 27.

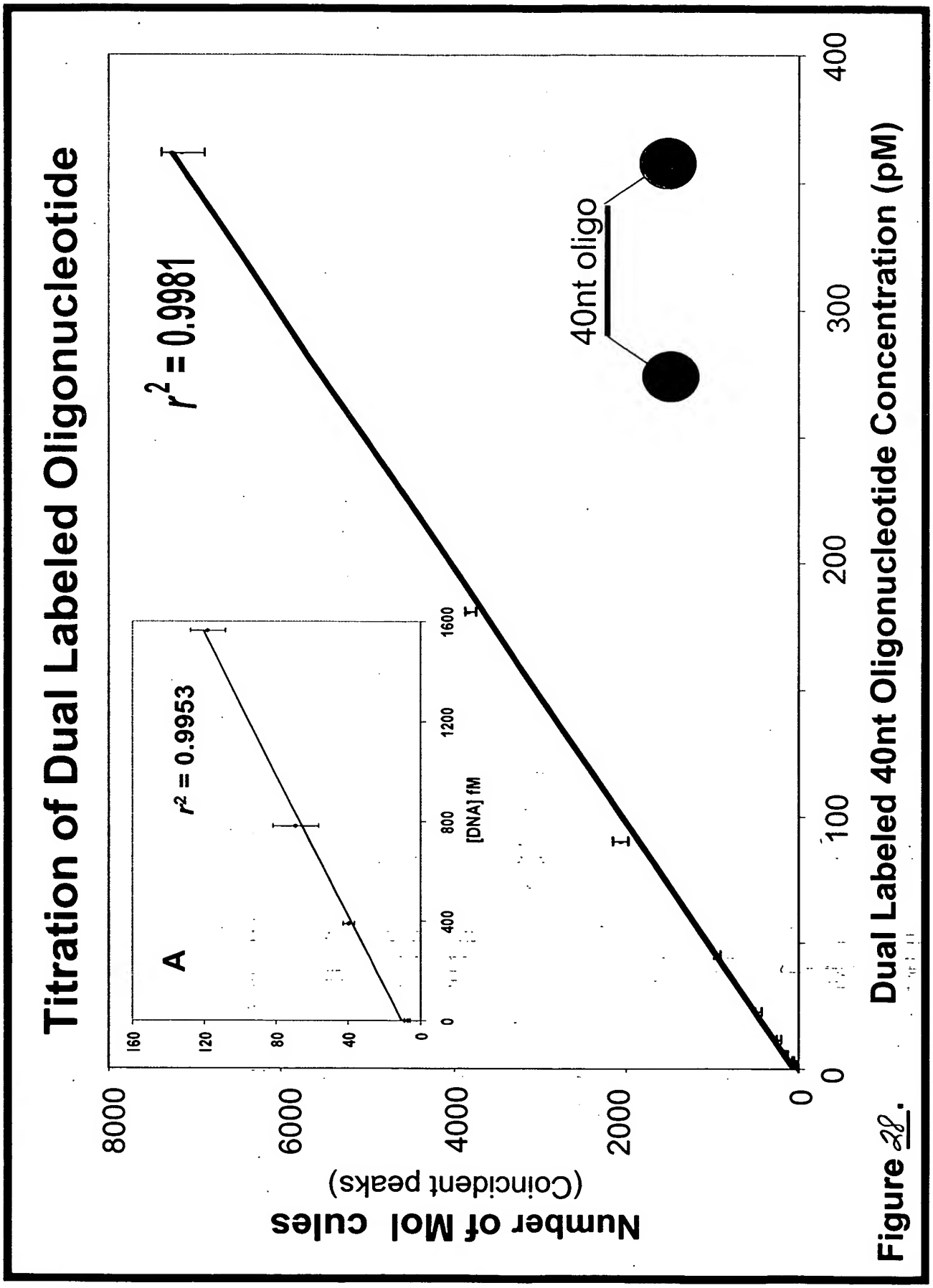

Titration of Dual Labeled Oligonucleotide
Number of Molecules
(Coincident peaks)
8000
6000
4000
2000
0
0
100
200
300
400
Dual Labeled 40nt Oligonucleotide Concentration (pM)
$r^2 = 0.9981$
40nt oligo
A
$r^2 = 0.9953$
160
120
80
40
0
0
400
800
1200
1600
[DNA] fM

Figure 2B.

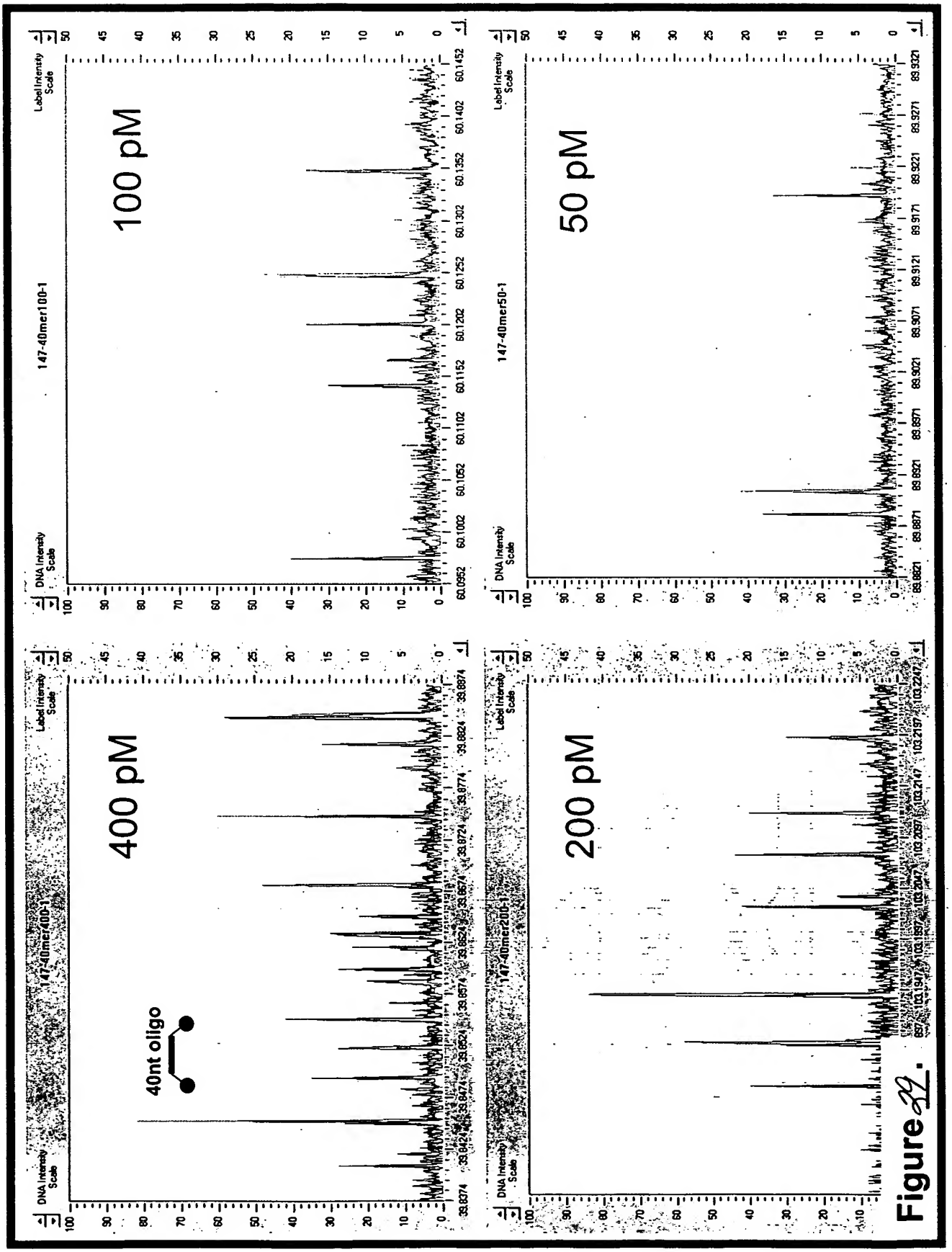
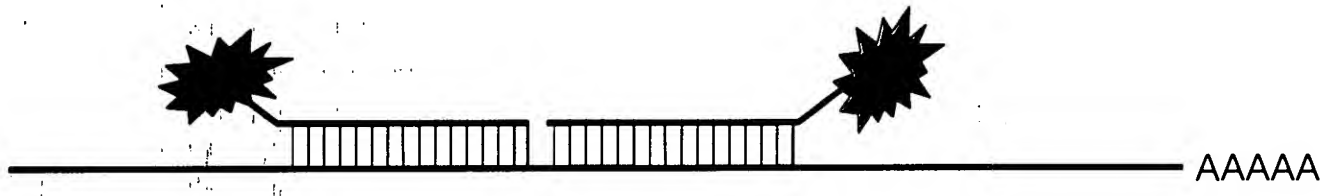

100 pM
50 pM
400 pM
200 pM
40nt oligo
DNA Intensity
Label Intensity
Scale
147-40mer100-1
147-40mer50-1
147-40mer400-1
147-40mer200-1

Figure 22.

Two Assays Validated

- ***Dual Probe Hybridization***: Hybridize two oligos (20 - 30 nt) to RNA target



- ***Probe Extension***: Hybridize one Cy5 labeled oligo and incorporate TAMRA by reverse transcription

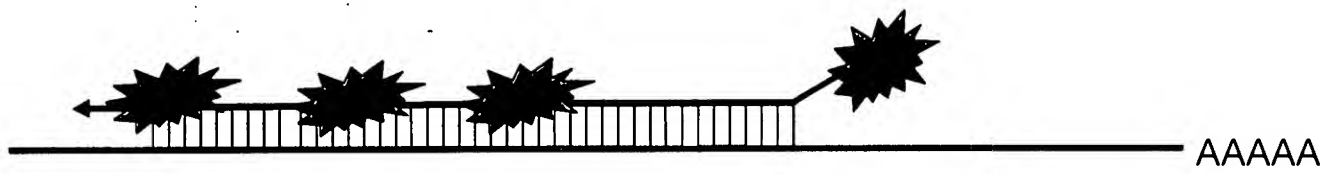
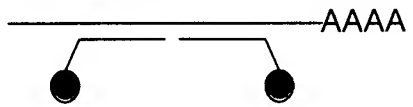


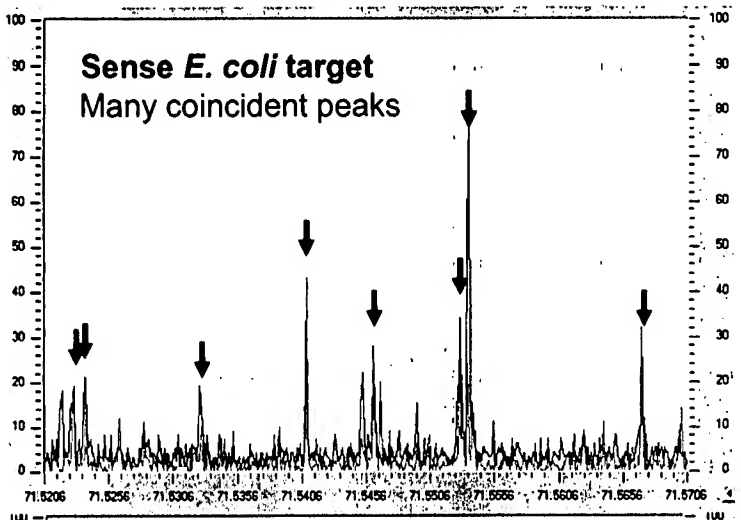
Figure 30.

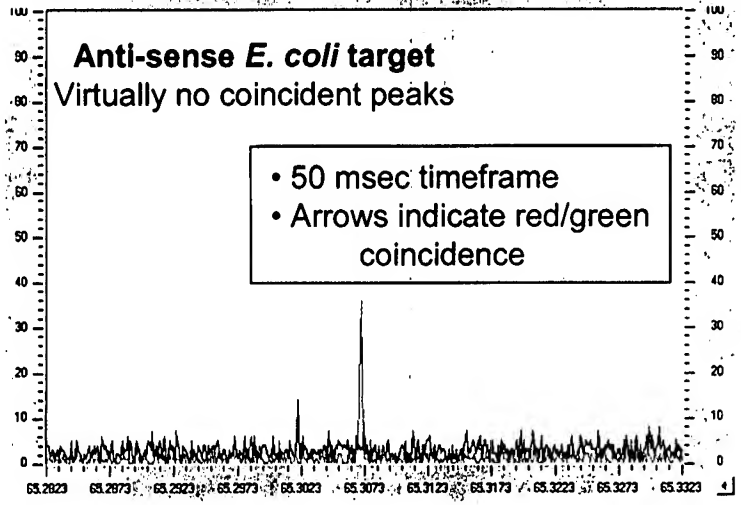
METHODS AND APPARATI USING SINGLE POLYMER ANALYSIS
Inventors: Zhao et al.
Serial No.: TBD
Docket No. C0989.70049US01

Dual Probe Hybridization



- Sense vs. Antisense *E.coli* spike in total human RNA background





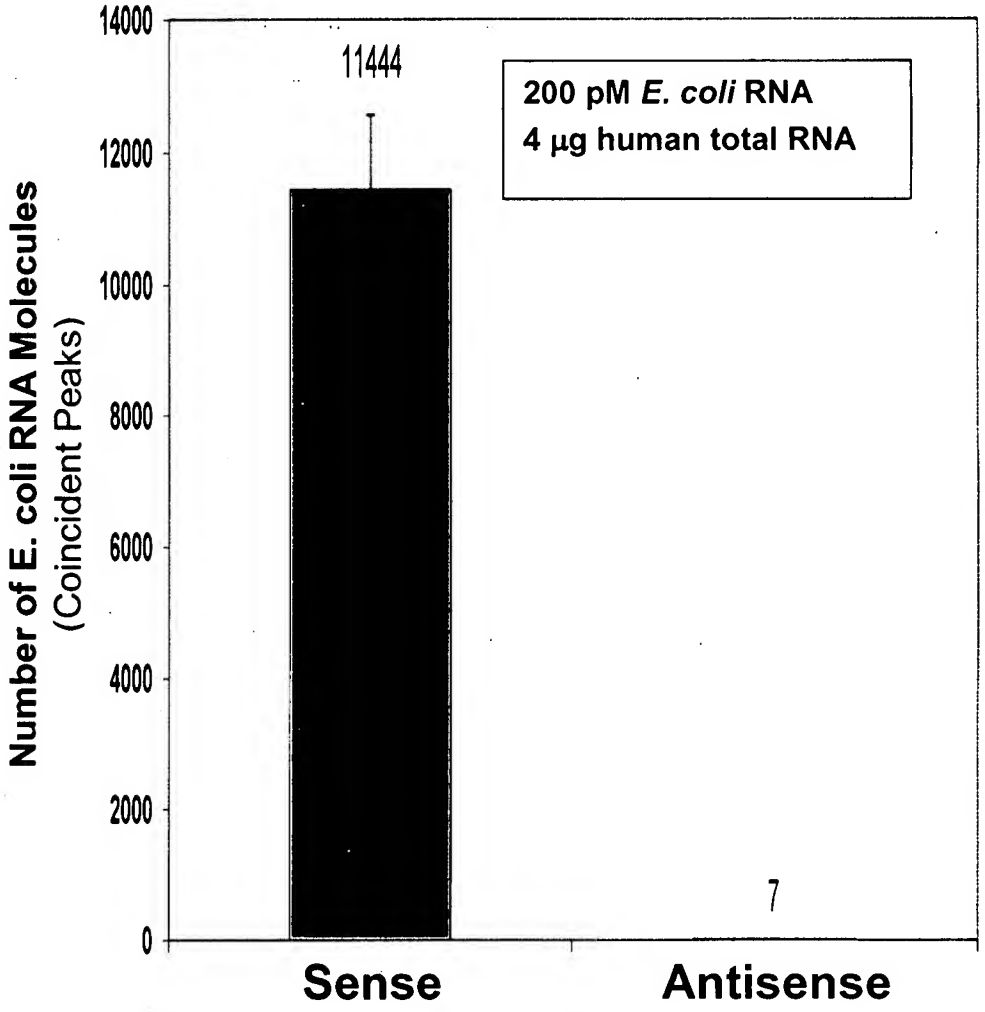
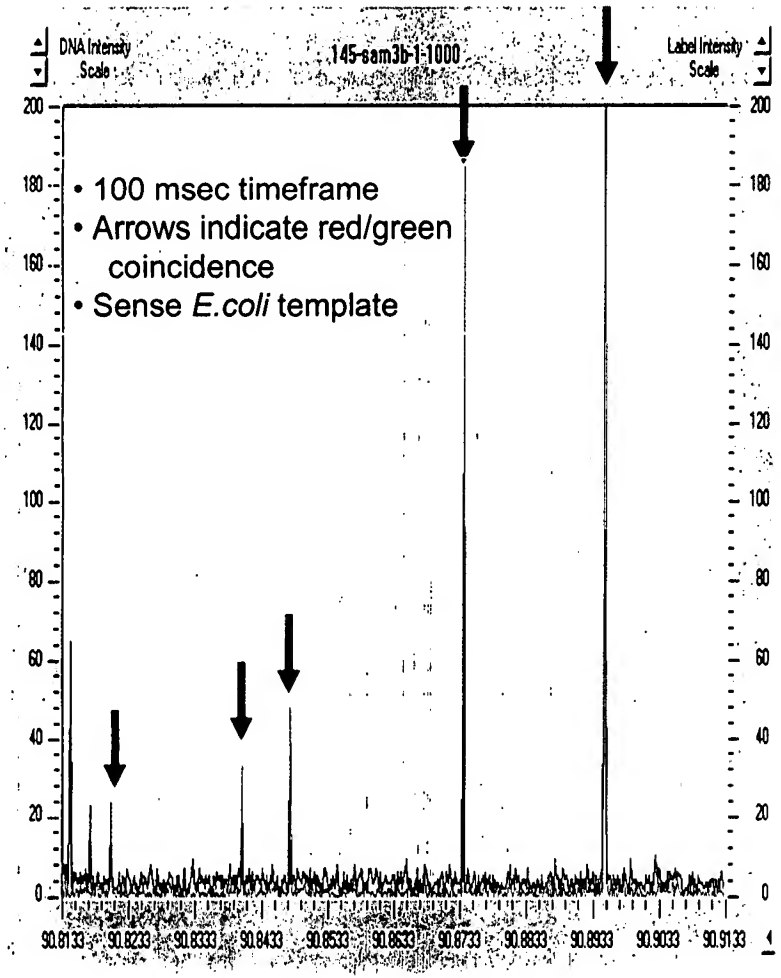
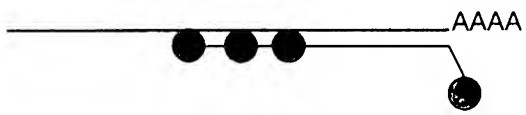
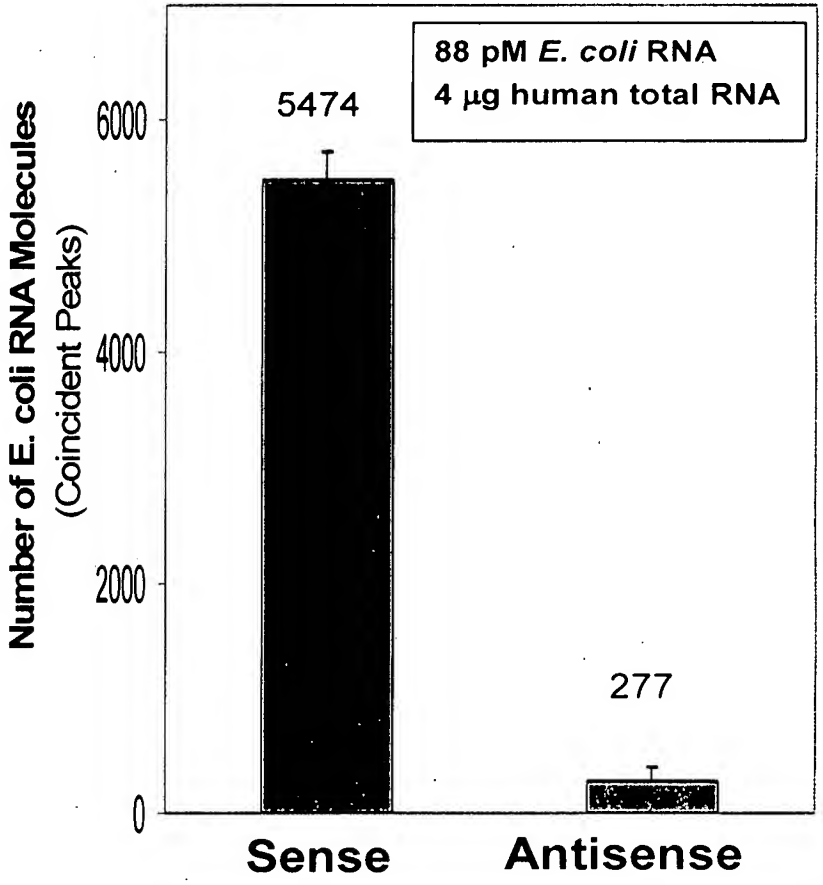


Figure 31.

Probe Extension Assay







Figur 32.

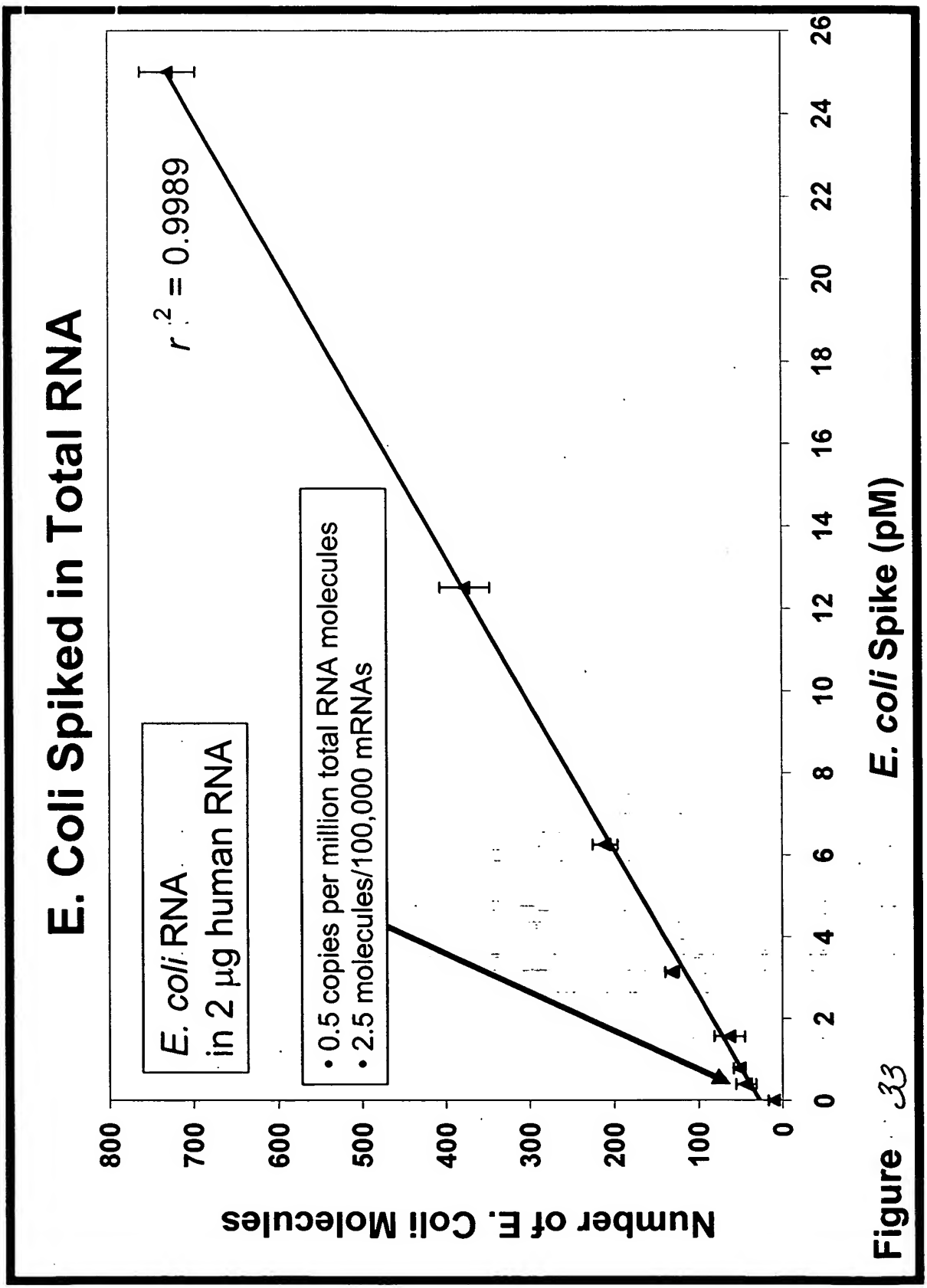

E. Coli Spiked in Total RNA
E. coli RNA in 2 µg human RNA
• 0.5 copies per million total RNA molecules
• 2.5 molecules/100,000 mRNAs
r^2 = 0.9989
Number of E. Coli Molecules
E. coli Spike (pM)
0
100
200
300
400
500
600
700
800
0
2
4
6
8
10
12
14
16
18
20
22
24
26

Figure 33

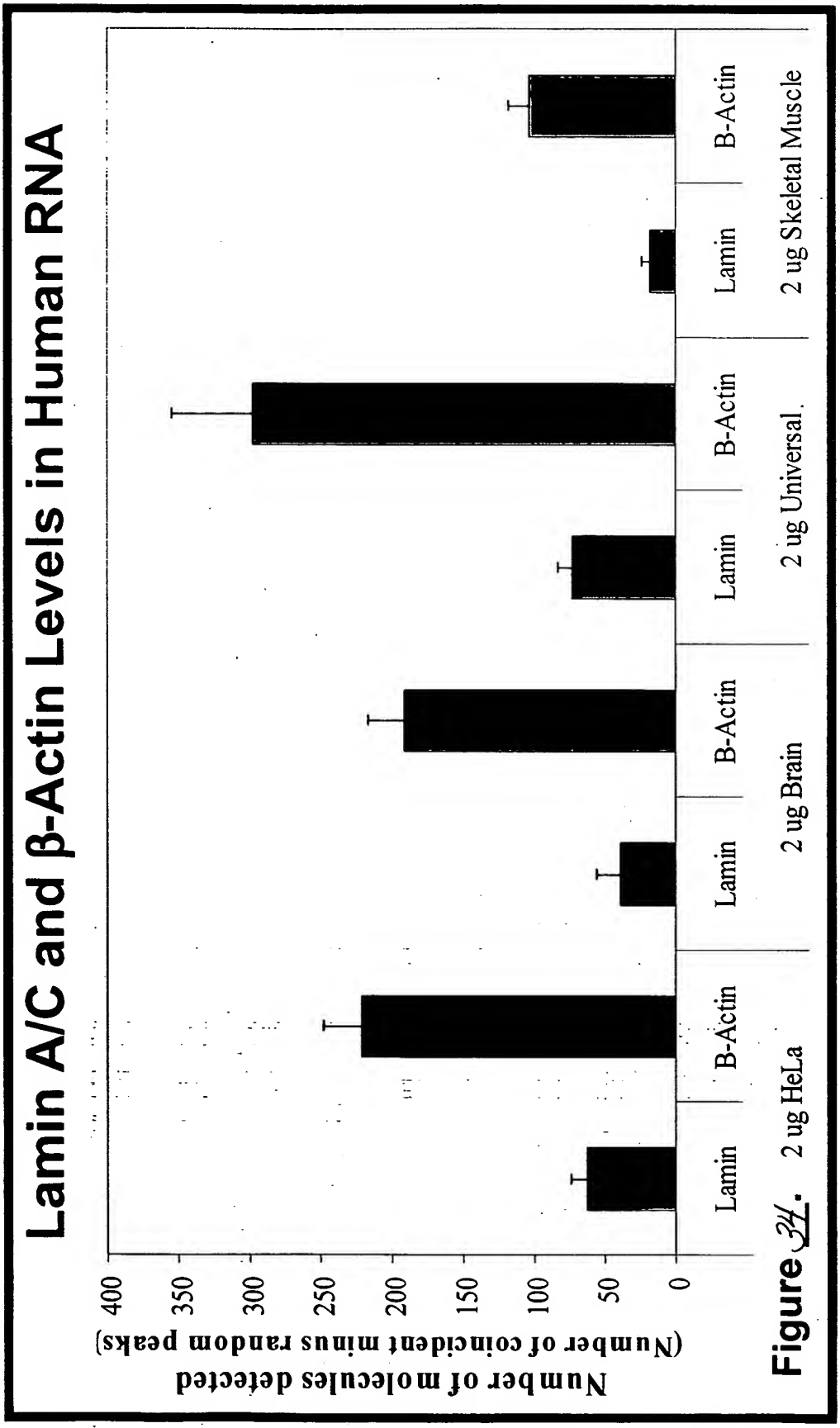

Lamin A/C and β-Actin Levels in Human RNA
Number of molecules detected
(Number of coincident minus random peaks)
400
350
300
250
200
150
100
50
0
Lamin
B-Actin
Lamin
B-Actin
Lamin
B-Actin
Lamin
B-Actin
2 ug HeLa
2 ug Brain
2 ug Universal
2 ug Skeletal Muscle

Figure 24.

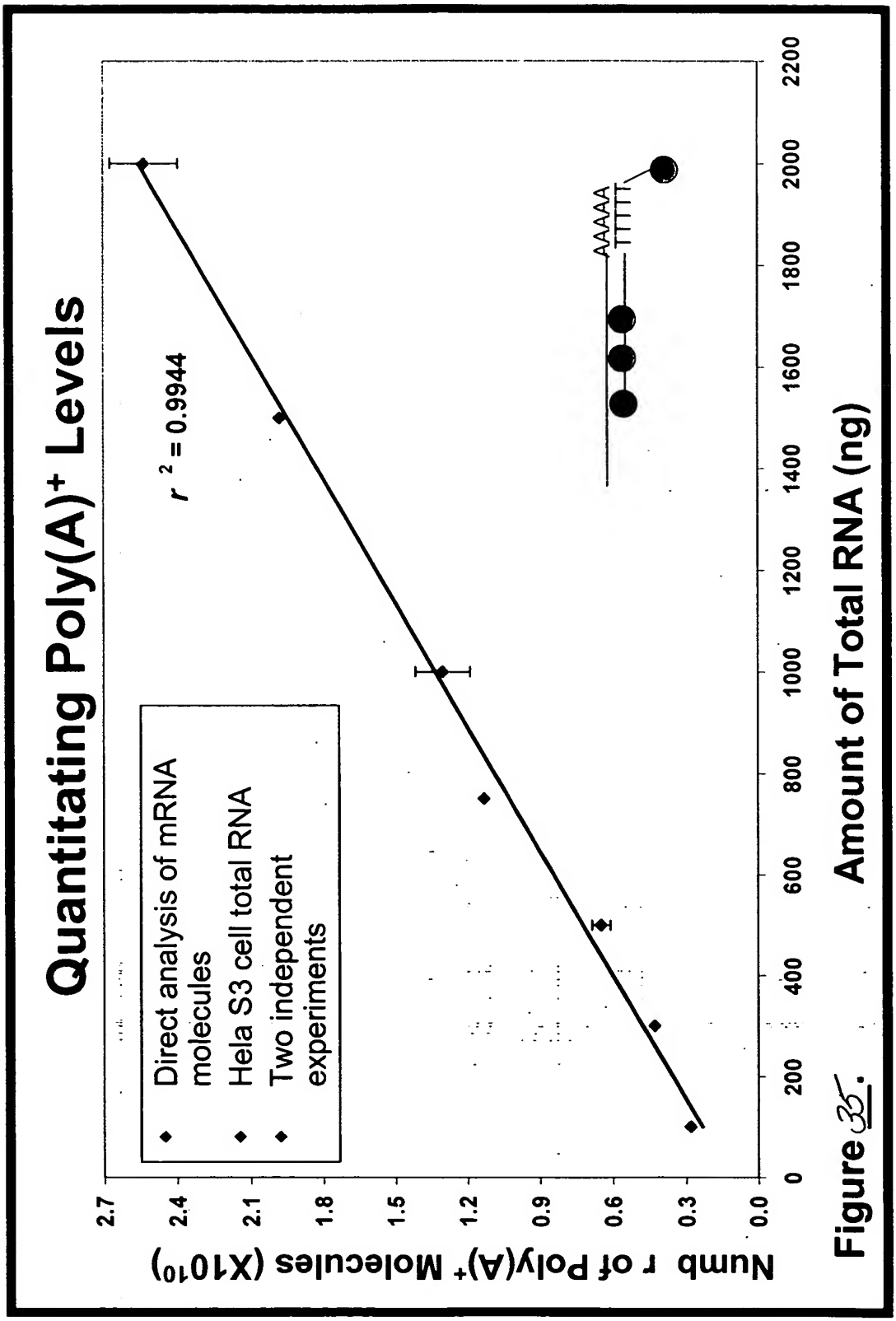
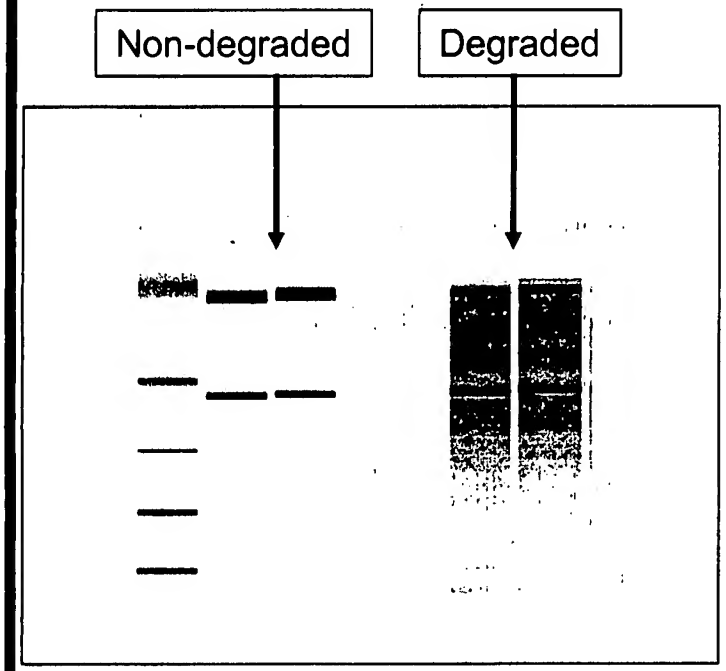

Quantitating Poly(A)+ Levels
Direct analysis of mRNA molecules
Hela S3 cell total RNA
Two independent experiments
r 2 = 0.9944
AAAAA
TTTTT
Numb r of Poly(A)+ Molecules (X1010)
Amount of Total RNA (ng)
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
2.7
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200

Figure 35.

Assessing mRNA Degradation

Total RNA Samples



Ratio of Green/Red Average Peak Areas by *DirectRNA*™

	G/R Avg. Peak Areas	Std Dev
Non-degraded RNA	2.64	0.02
Degraded RNA	0.88	0.10
Dual-labeled 40 mer	0.79	0.02

Figure 36.

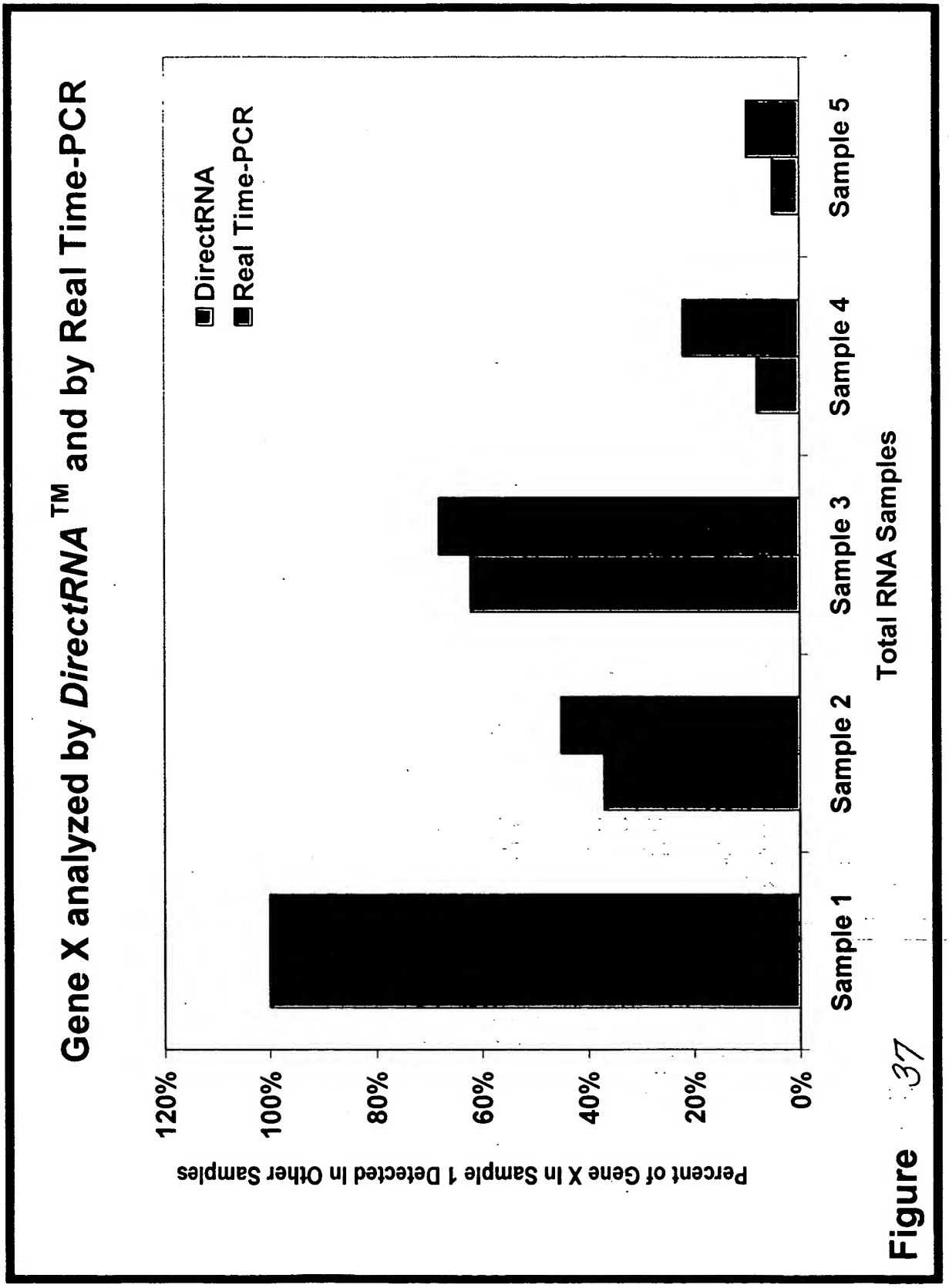
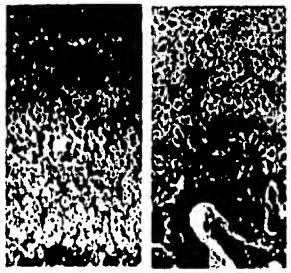

Gene X analyzed by DirectRNA™ and by Real Time-PCR
DirectRNA
Real Time-PCR
Percent of Gene X In Sample 1 Detected In Other Samples
120%
100%
80%
60%
40%
20%
0%
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Total RNA Samples

Figure 37

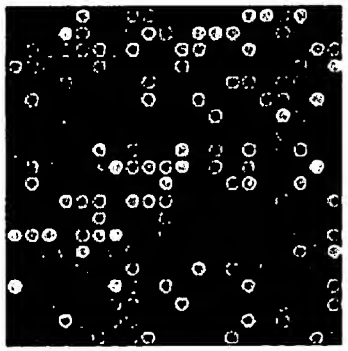
Quantitation in RNA Analysis by DirectRNA™

INPUT



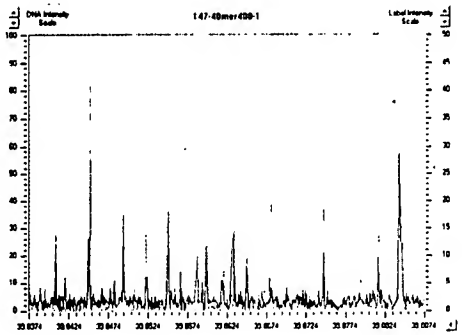
Disease Sample **Control Sample**

TOOLS & ANALYSES



Microarray analysis

First-pass analysis across thousands of genes



Quantitative Analysis DirectRNA™

Detailed analysis of specific genes of interest

OUTPUT

- Disease genes
- Pathways
- Drug targets

Figure 38.

Fig. 39A

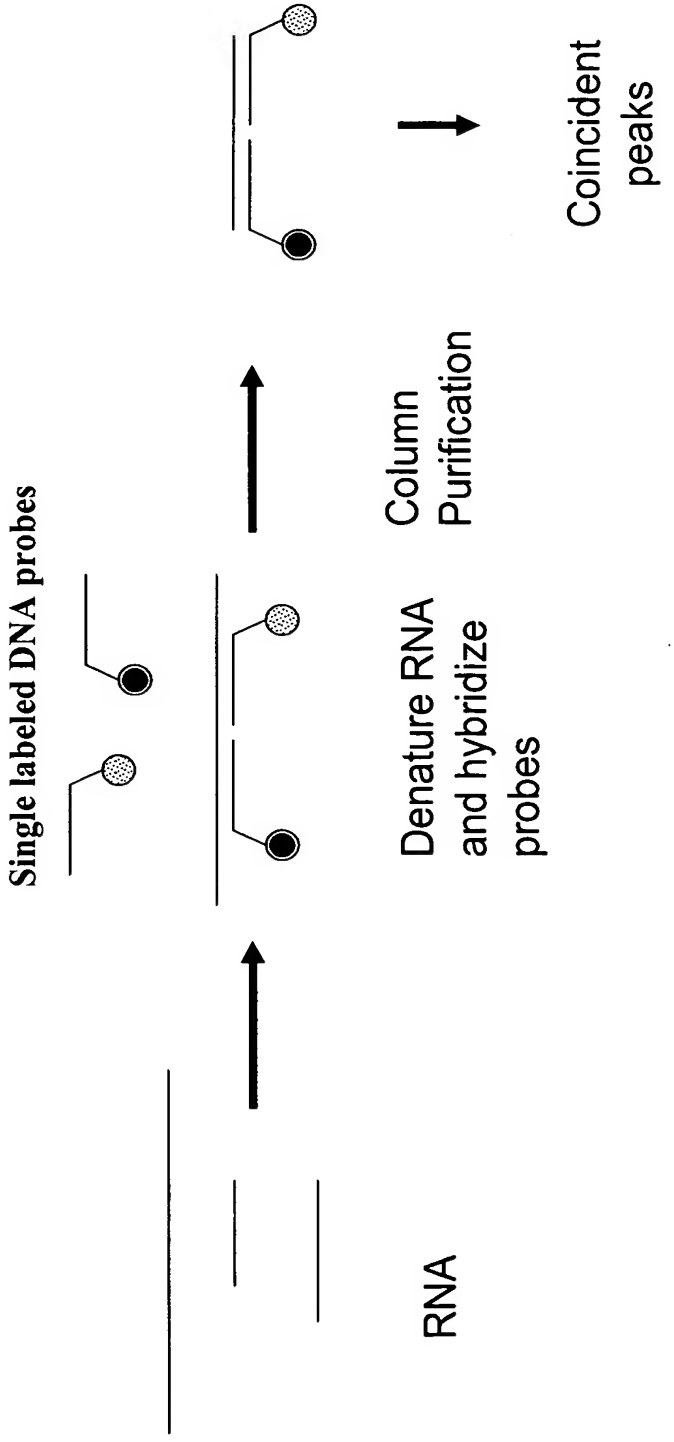

Single labeled DNA probes
RNA
Denature RNA and hybridize probes
Column Purification
Coincident peaks

Fig. 39B

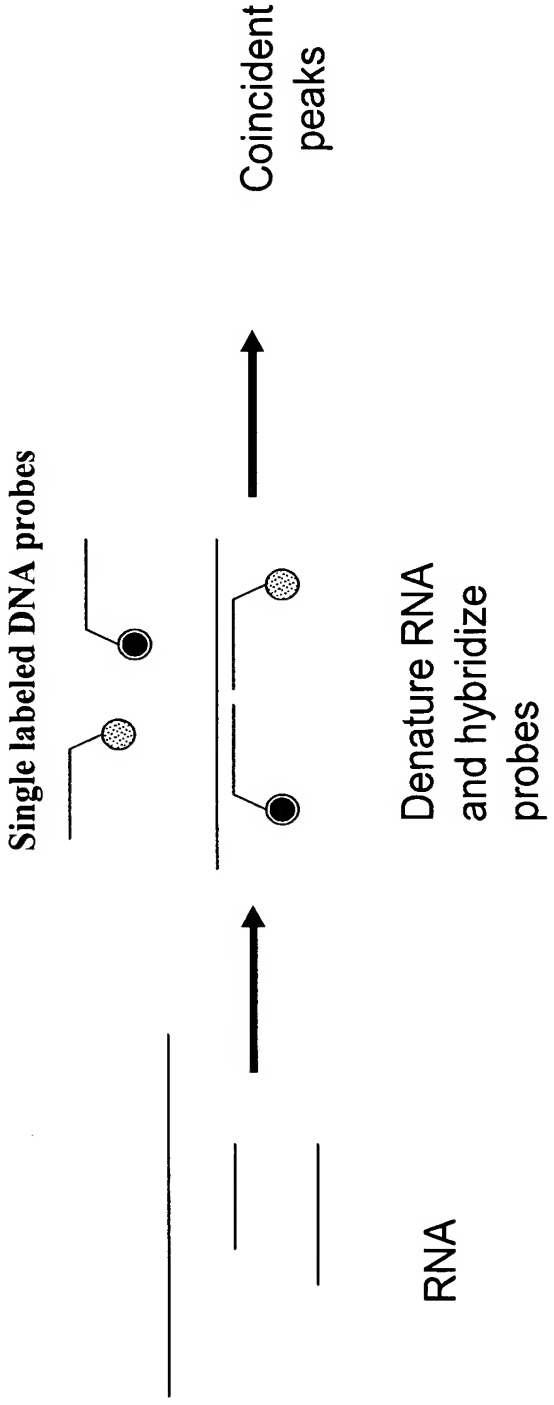

Single labeled DNA probes
Denature RNA and hybridize probes
RNA
Coincident peaks

Fig. 40

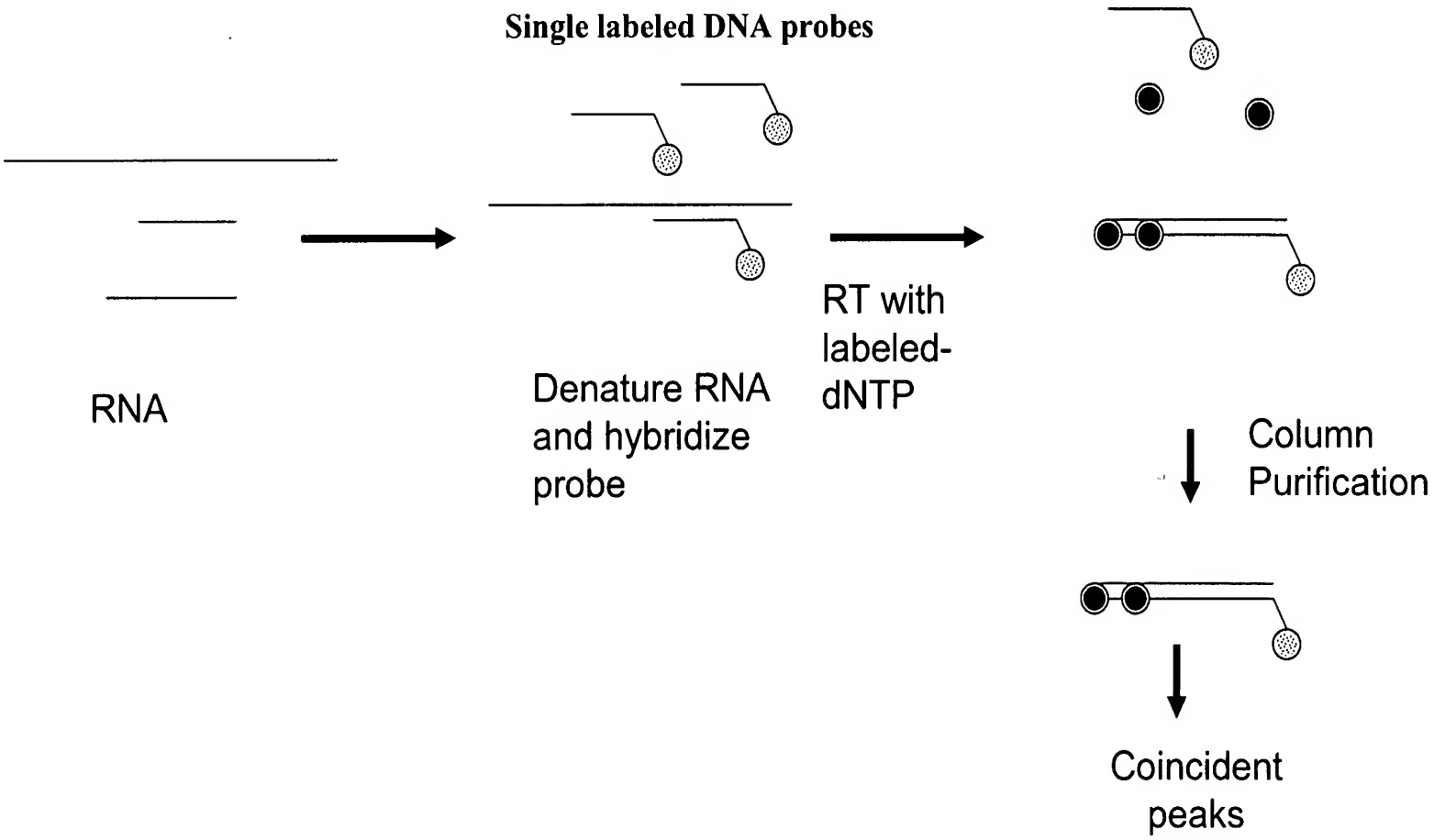

Single labeled DNA probes
RNA
Denature RNA and hybridize probe
RT with labeled-dNTP
Column Purification
Coincident peaks

Fig. 41A

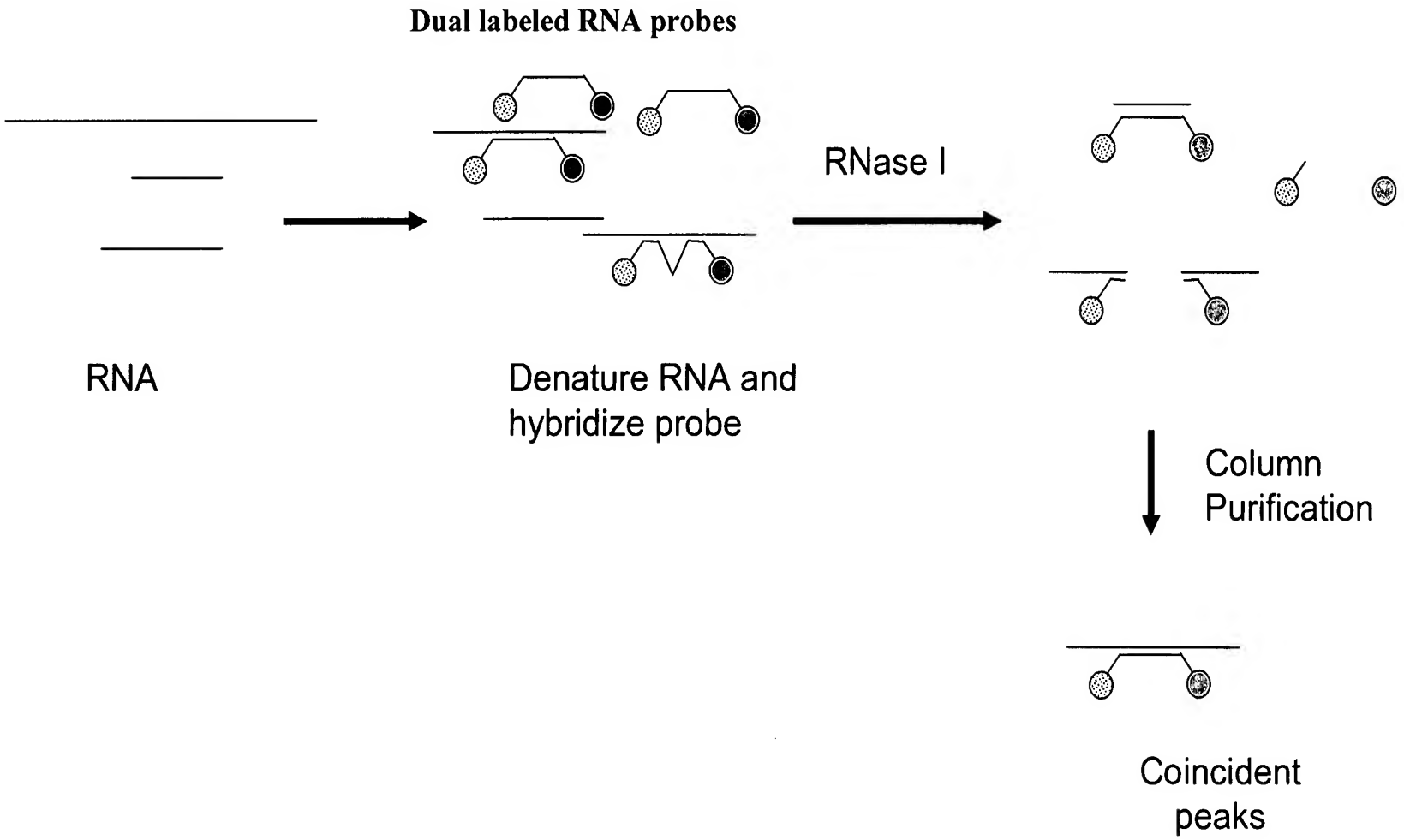

Dual labeled RNA probes
RNase I
RNA
Denature RNA and
hybridize probe
Column
Purification
Coincident
peaks

Fig. 41B

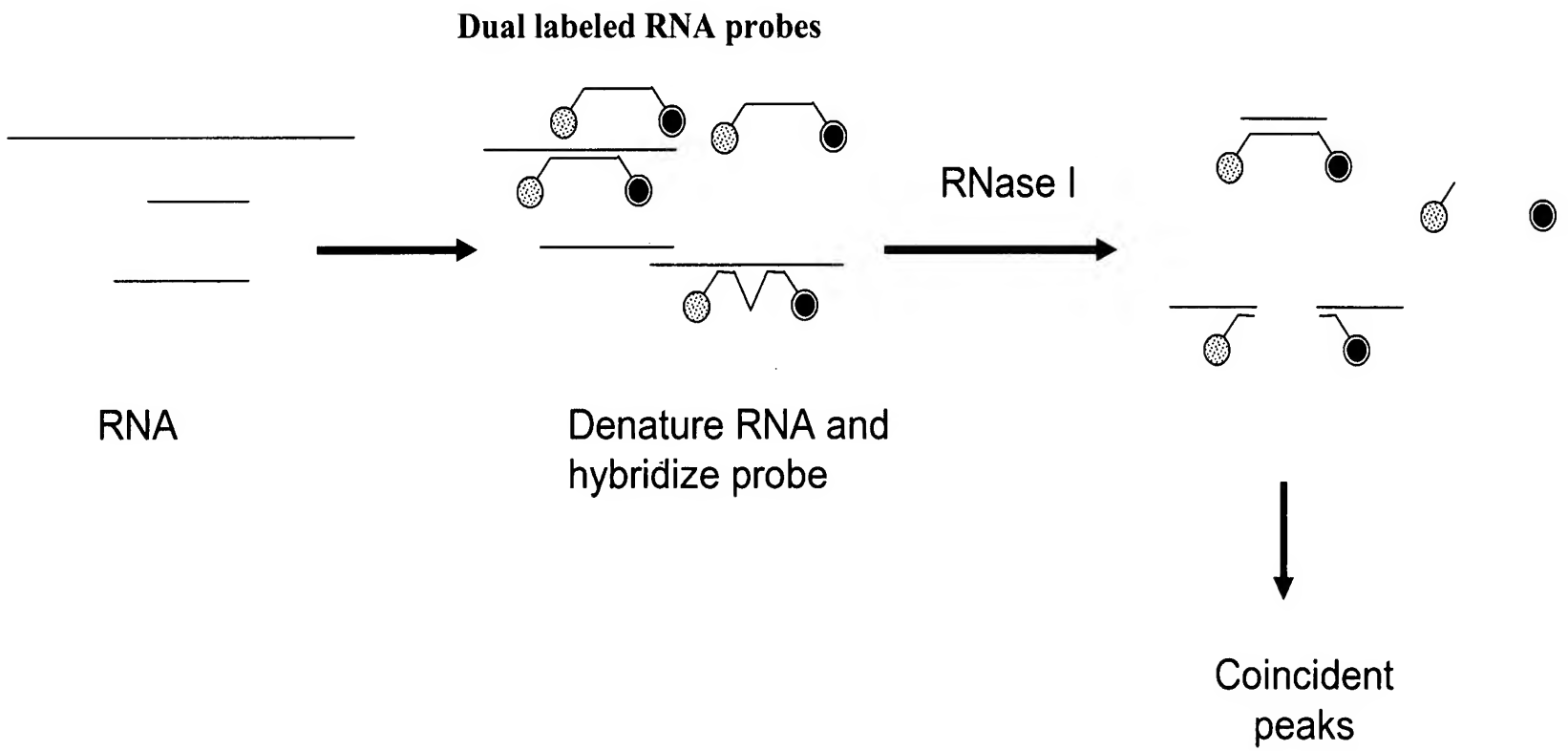

Dual labeled RNA probes
RNase I
RNA
Denature RNA and
hybridize probe
Coincident
peaks

Fig. 42A

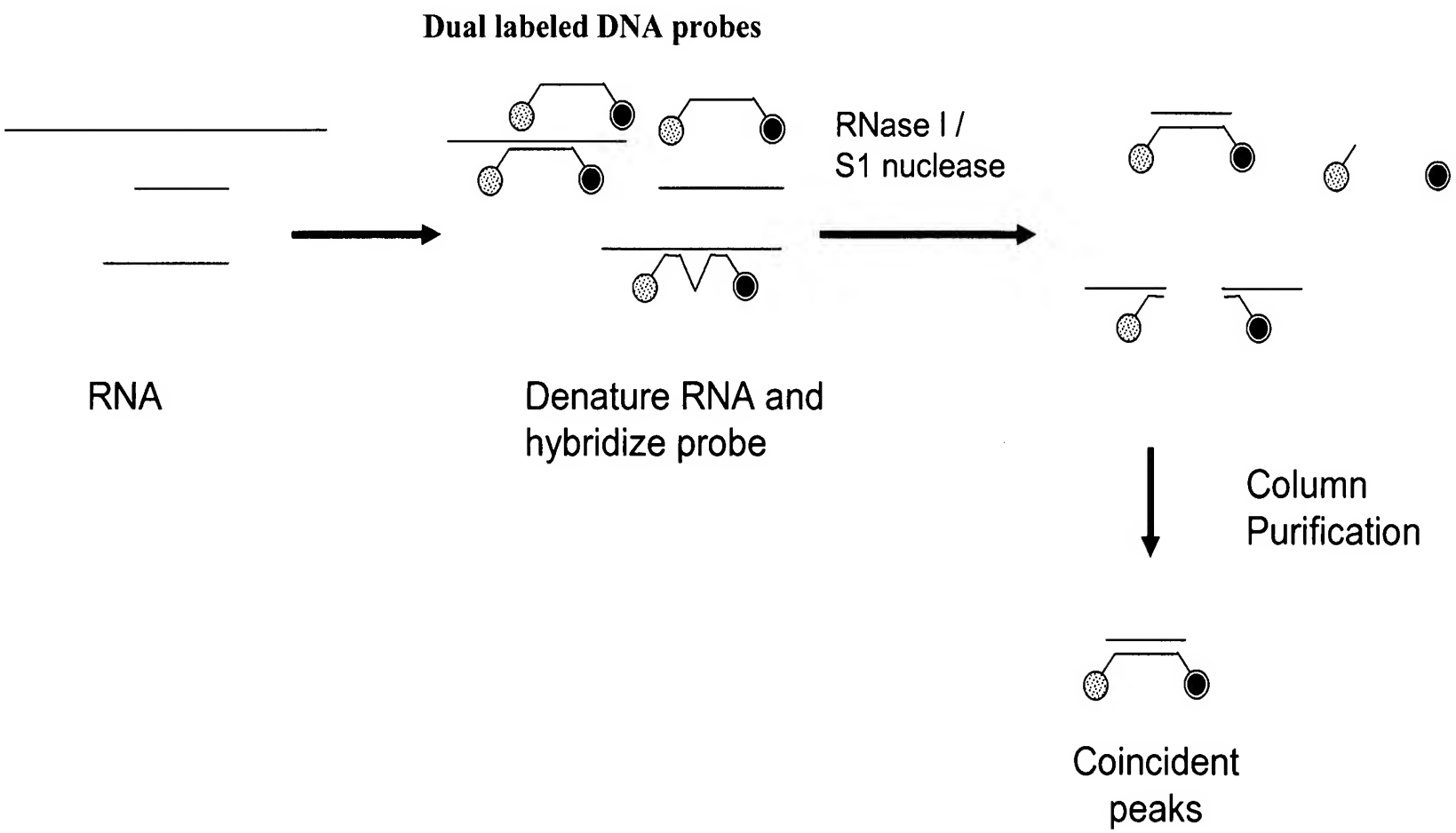

Dual labeled DNA probes
RNase I /
S1 nuclease
RNA
Denature RNA and
hybridize probe
Column
Purification
Coincident
peaks

Fig. 42B

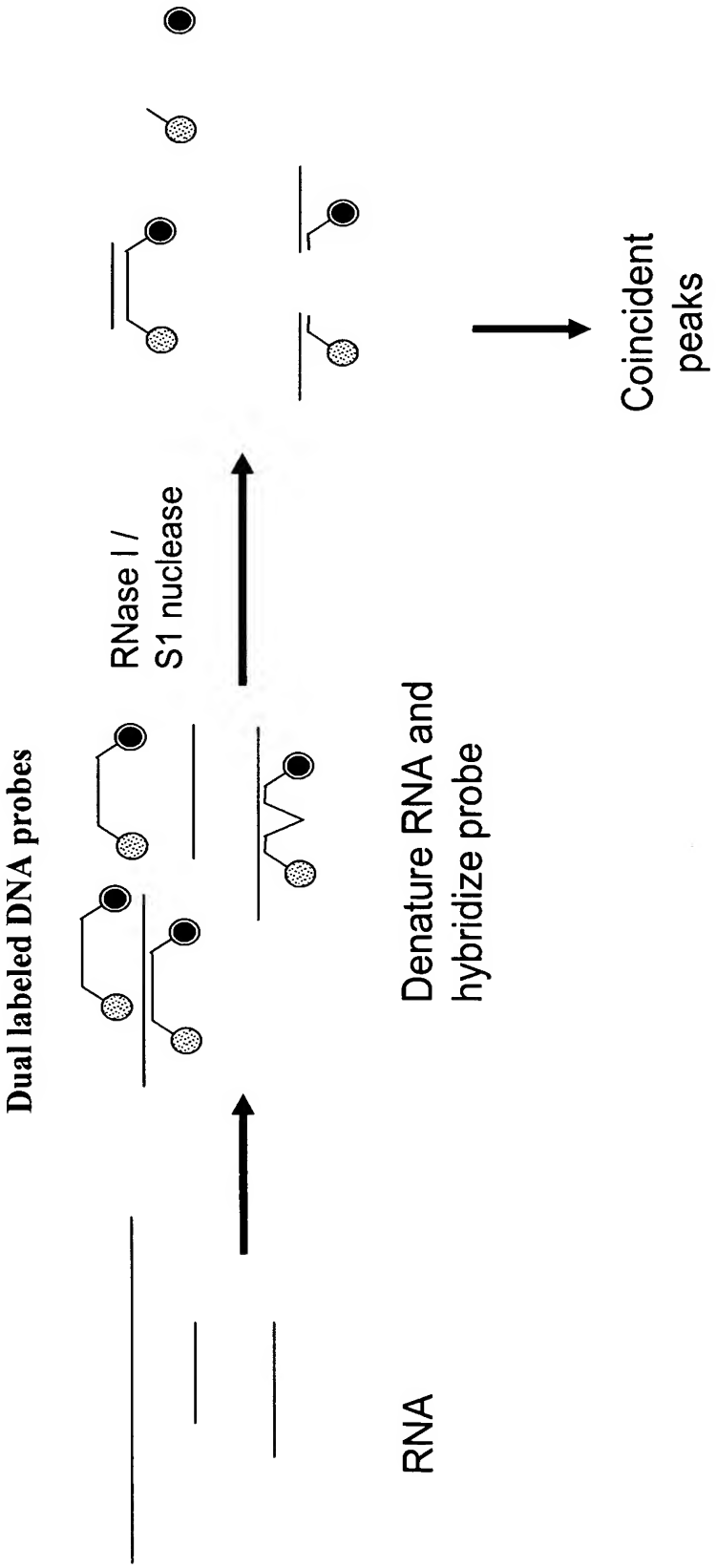

Dual labeled DNA probes
RNase I / S1 nuclease
Denature RNA and hybridize probe
RNA
Coincident peaks

Fig. 43

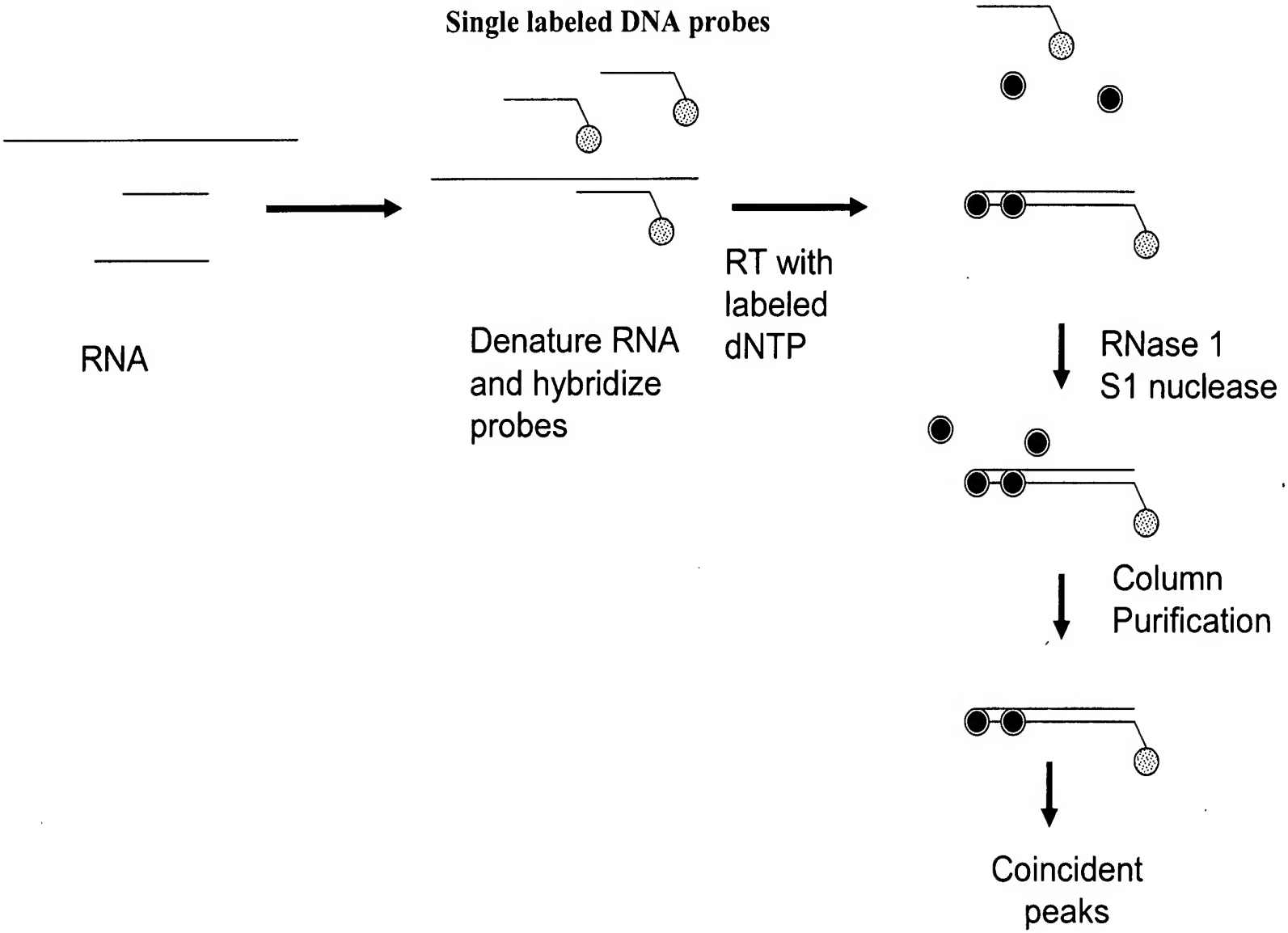

Single labeled DNA probes
RNA
Denature RNA and hybridize probes
RT with labeled dNTP
RNase 1 S1 nuclease
Column Purification
Coincident peaks

Fig. 44

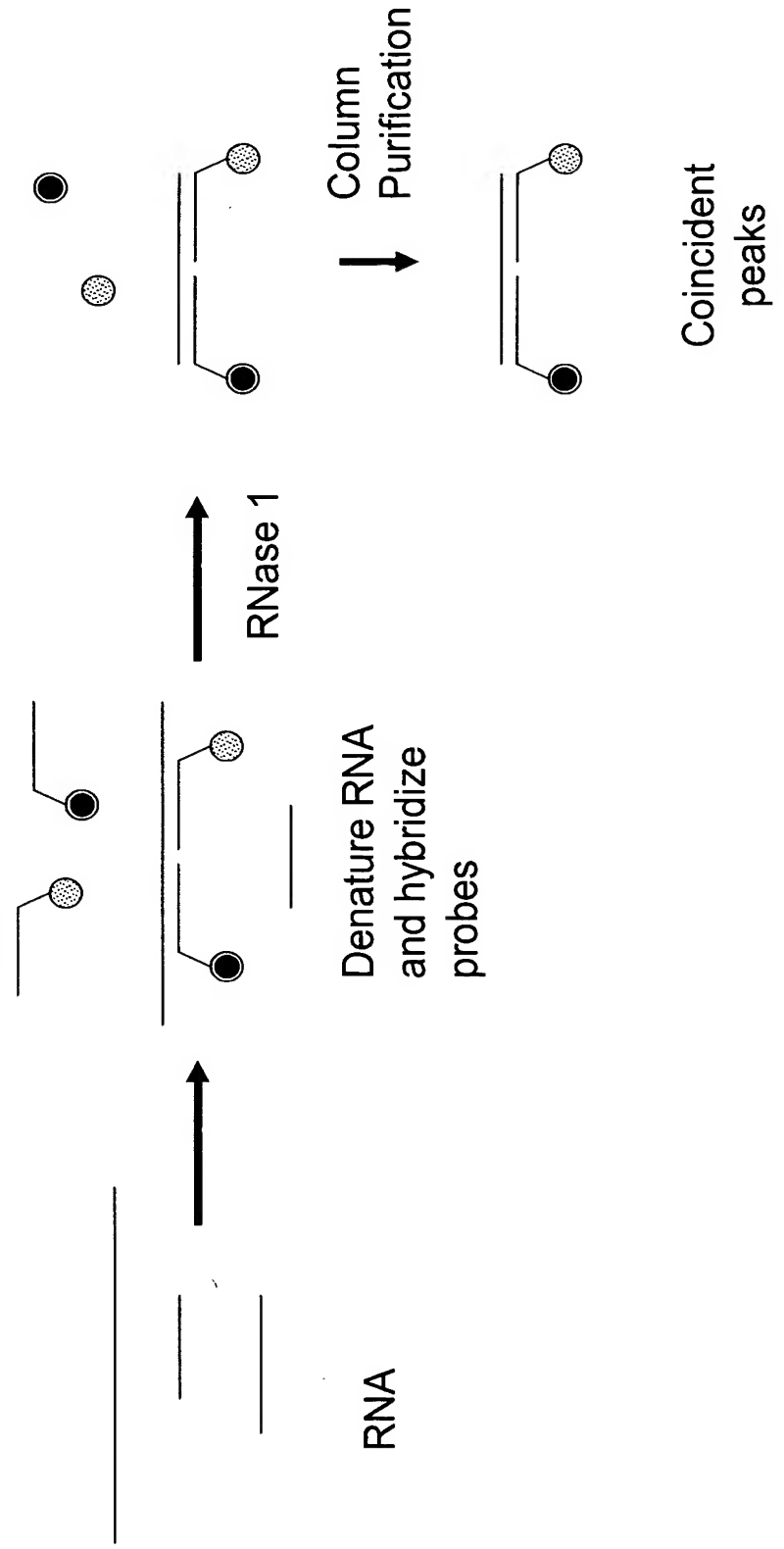

RNA
Single labeled RNA probes
Denature RNA and hybridize probes
RNase 1
Column Purification
Coincident peaks

Fig. 45

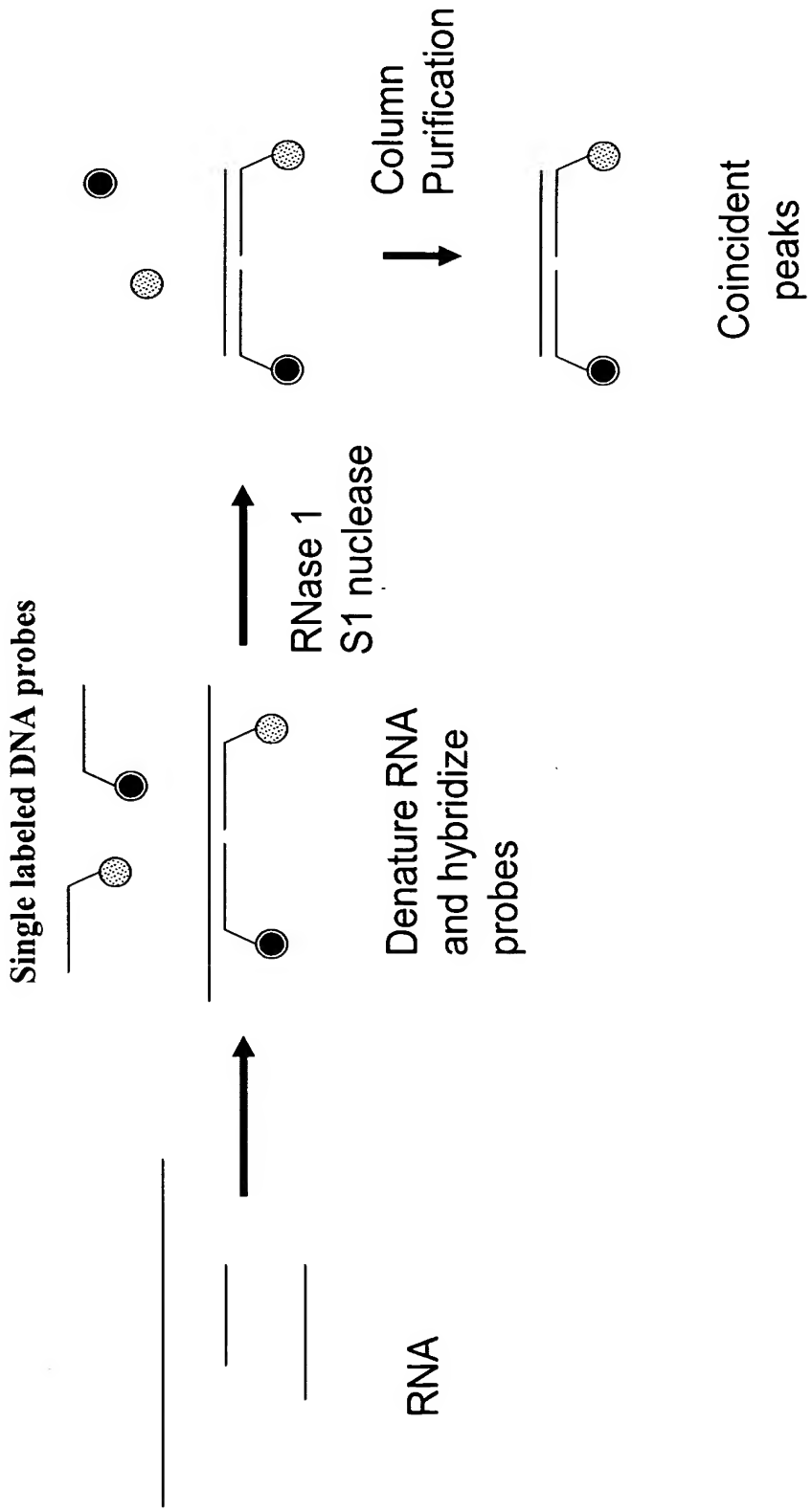

Single labeled DNA probes
RNA
Denature RNA and hybridize probes
RNase 1
S1 nuclease
Column Purification
Coincident peaks

Fig. 46

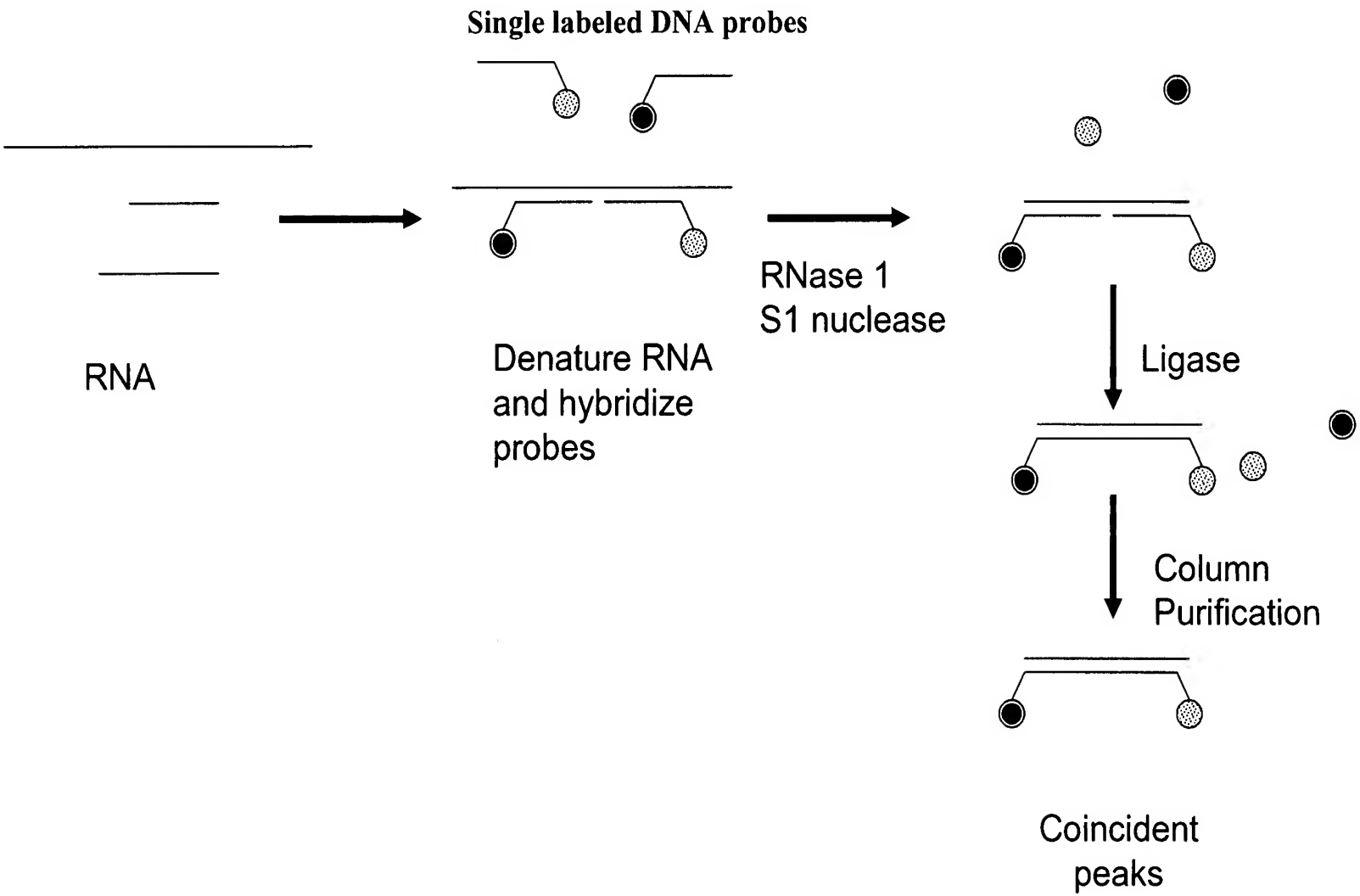

Single labeled DNA probes
RNA
Denature RNA and hybridize probes
RNase 1 S1 nuclease
Ligase
Column Purification
Coincident peaks

Fig. 47

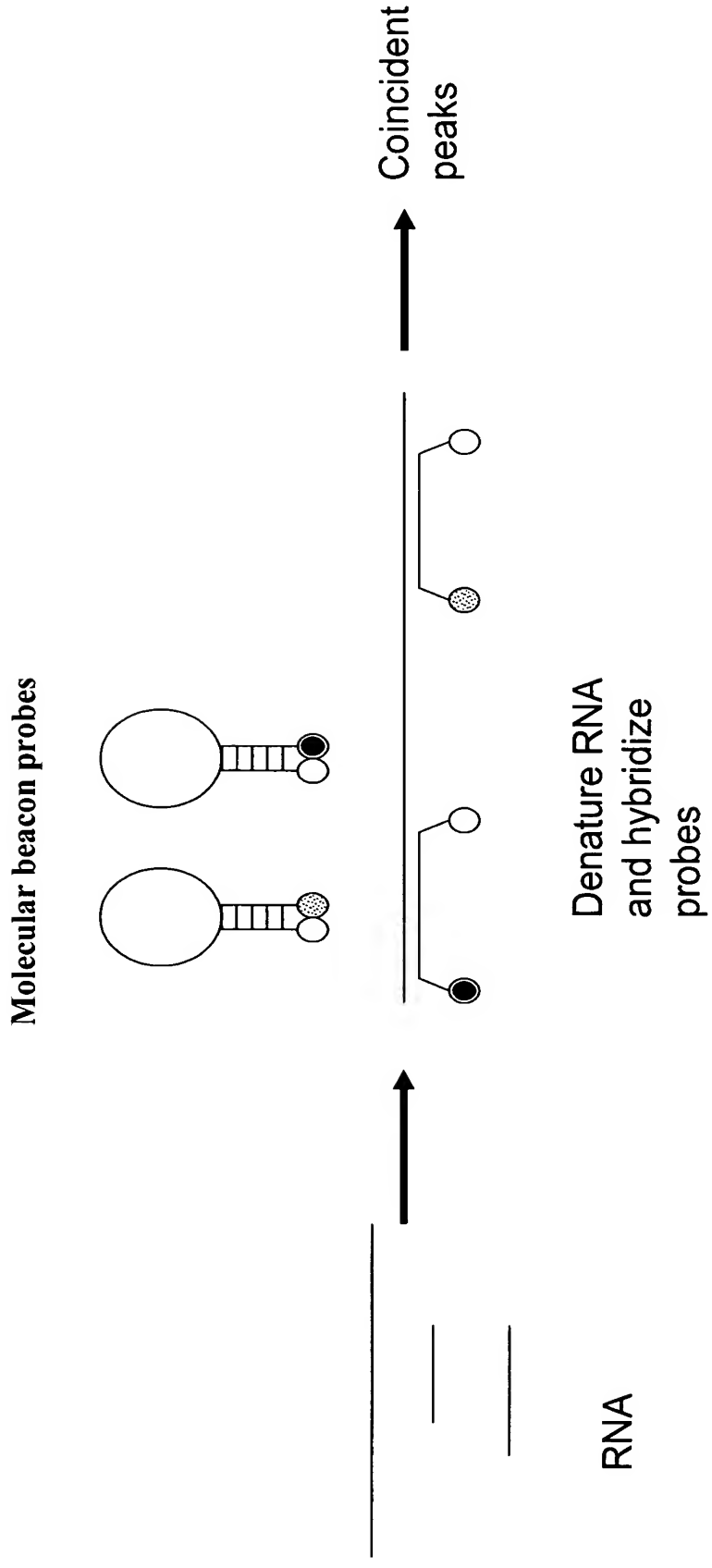

Molecular beacon probes
Denature RNA
and hybridize
probes
RNA
Coincident
peaks

Fig. 48A

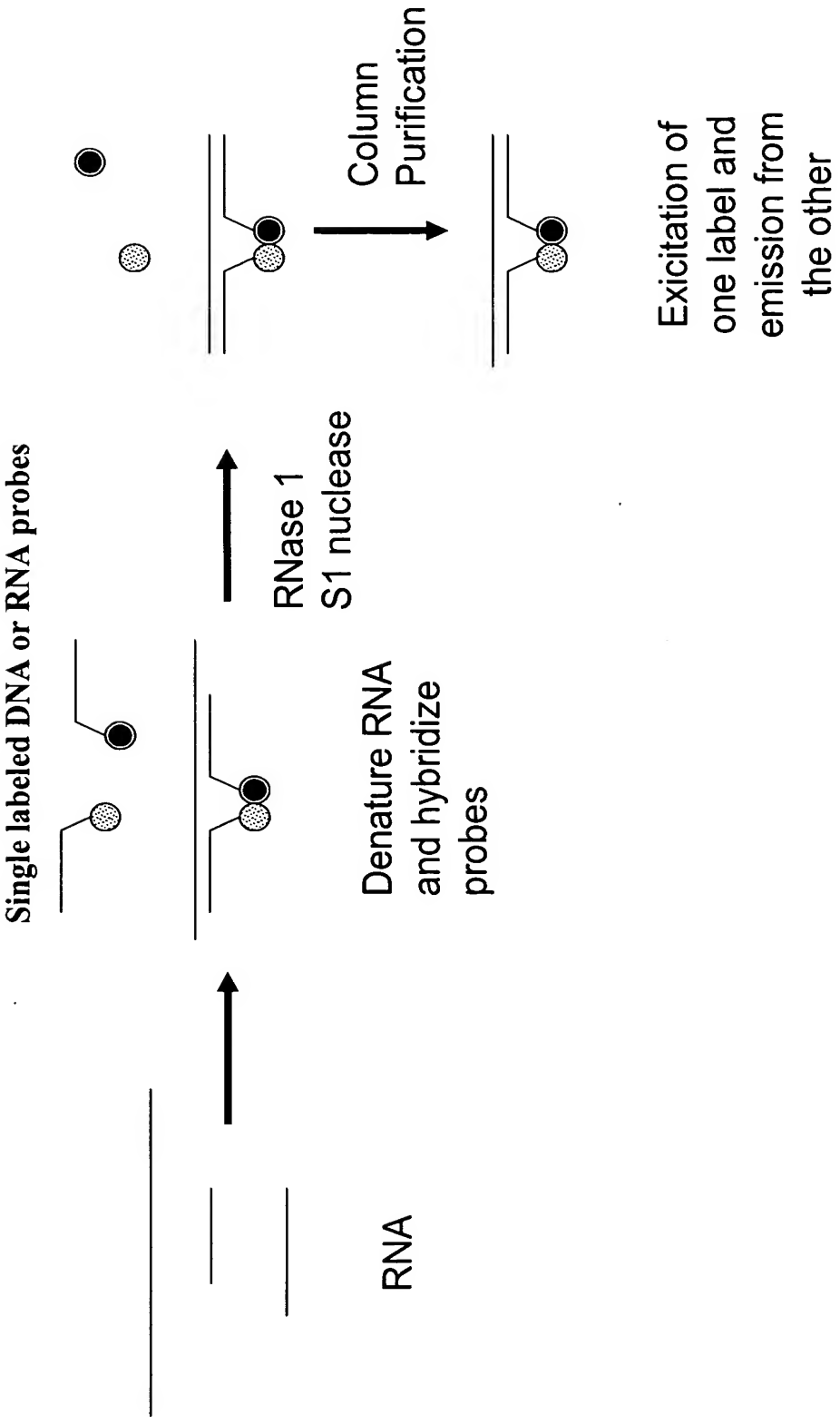

Single labeled DNA or RNA probes
RNA
Denature RNA and hybridize probes
RNase 1
S1 nuclease
Column Purification
Exicitation of one label and emission from the other

Fig. 4B

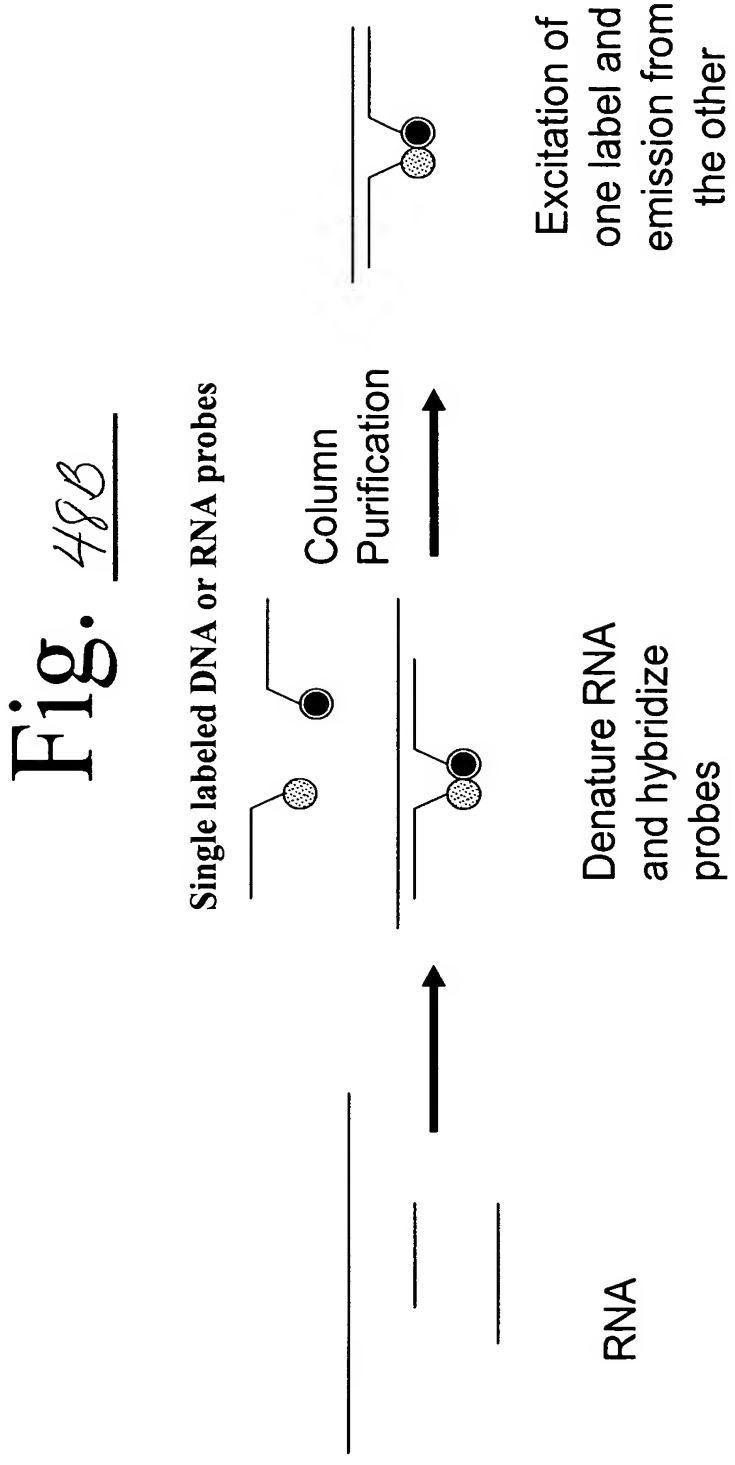

RNA
Denature RNA and hybridize probes
Single labeled DNA or RNA probes
Column Purification
Excitation of one label and emission from the other

Fig. 49

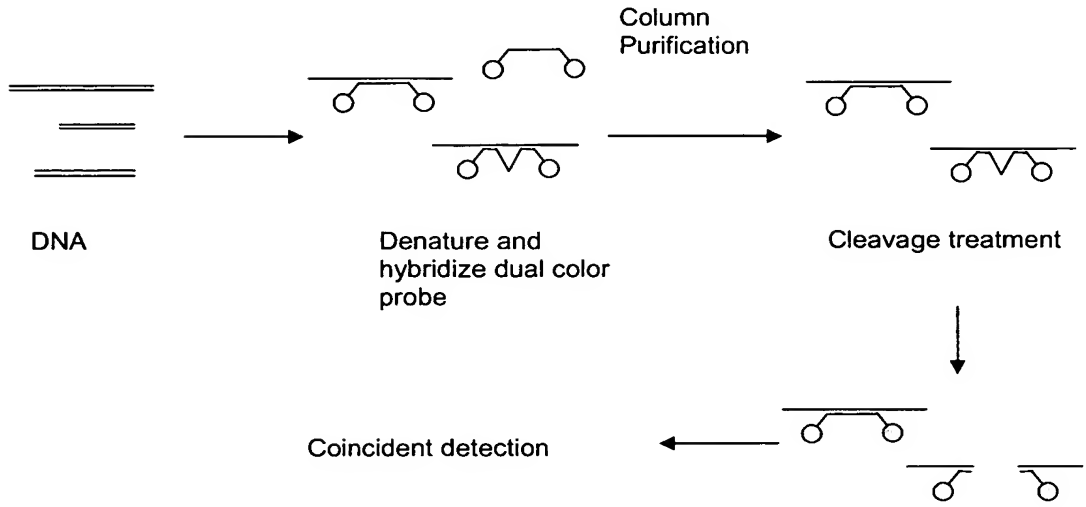

Column
Purification
DNA
Denature and
hybridize dual color
probe
Cleavage treatment
Coincident detection

Fig. 50

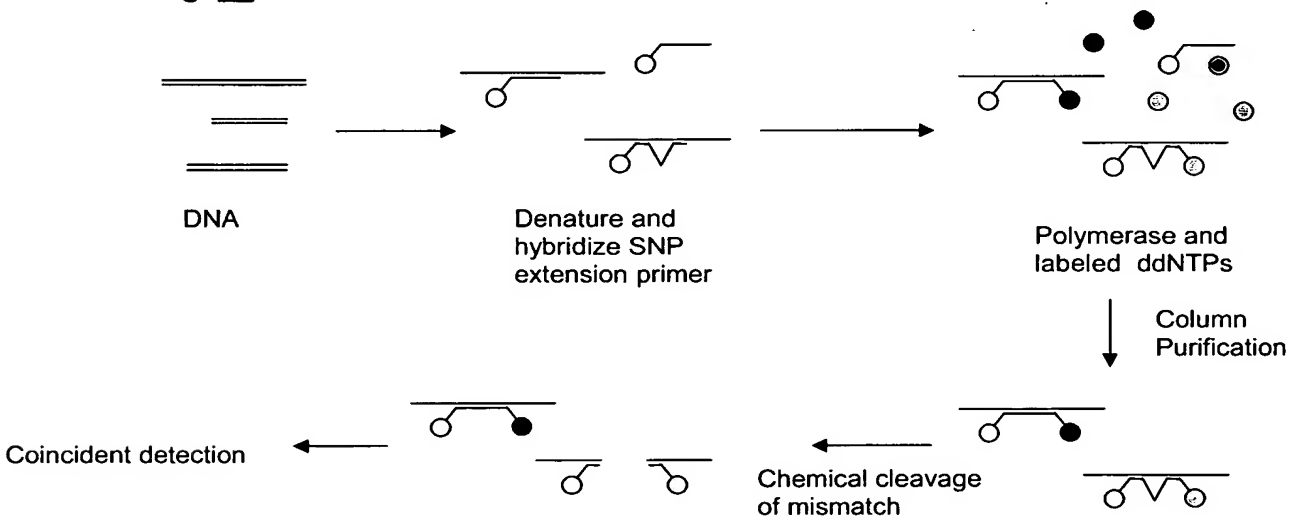

DNA
Denature and
hybridize SNP
extension primer
Polymerase and
labeled ddNTPs
Column
Purification
Coincident detection
Chemical cleavage
of mismatch

Fig. 51

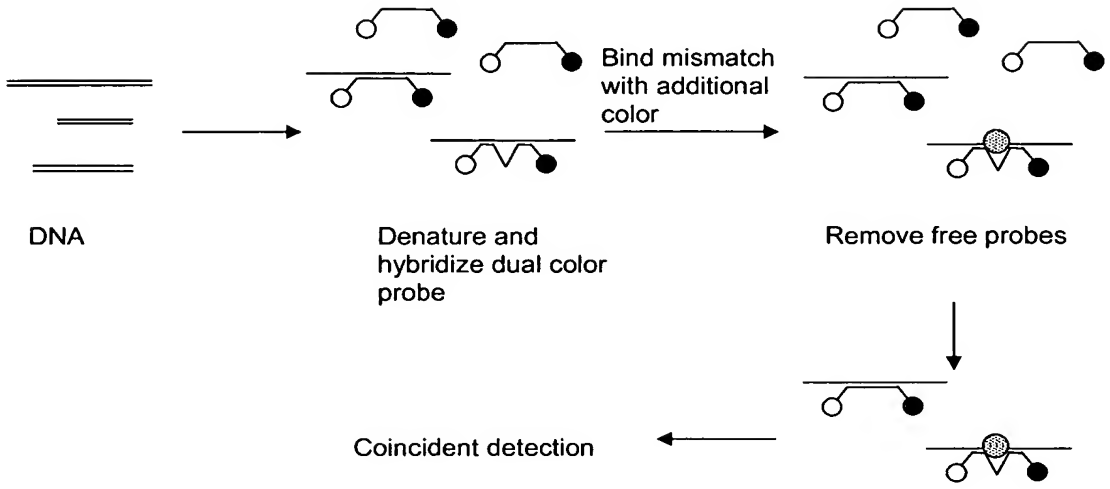

Bind mismatch with additional color
DNA
Denature and hybridize dual color probe
Remove free probes
Coincident detection

Fig. 52

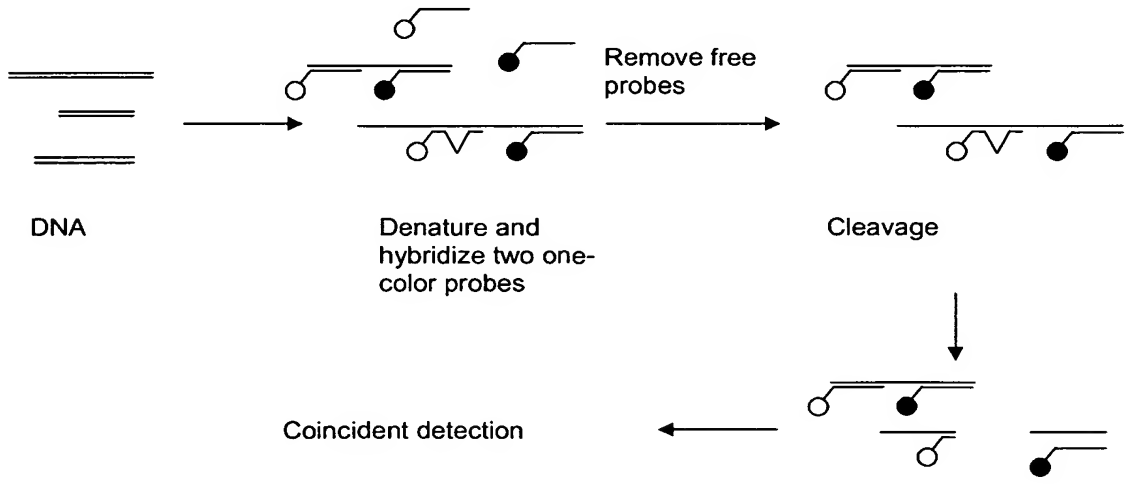

Remove free probes
DNA
Denature and hybridize two one-color probes
Cleavage
Coincident detection

Fig. 53

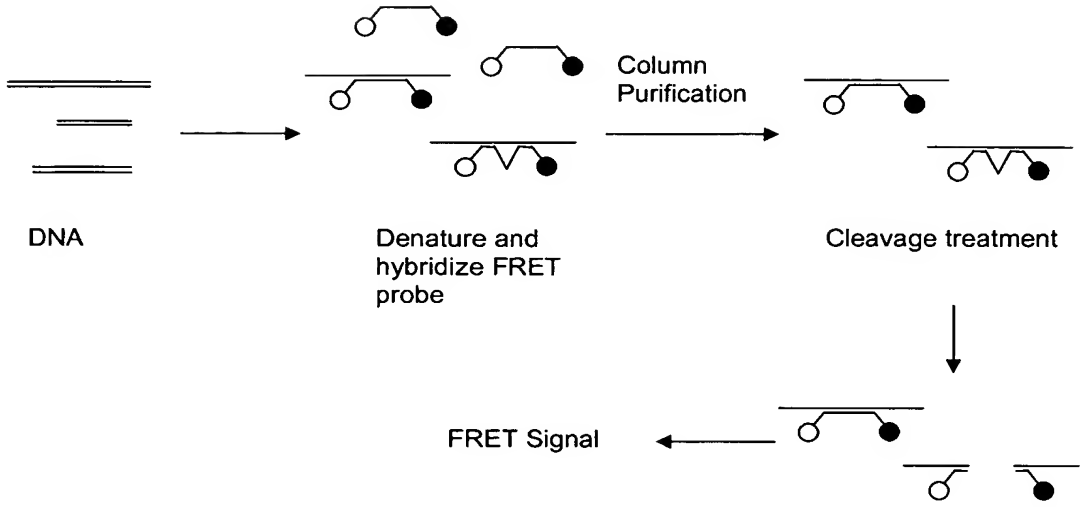

Column Purification
DNA
Denature and hybridize FRET probe
Cleavage treatment
FRET Signal

Fig. 54

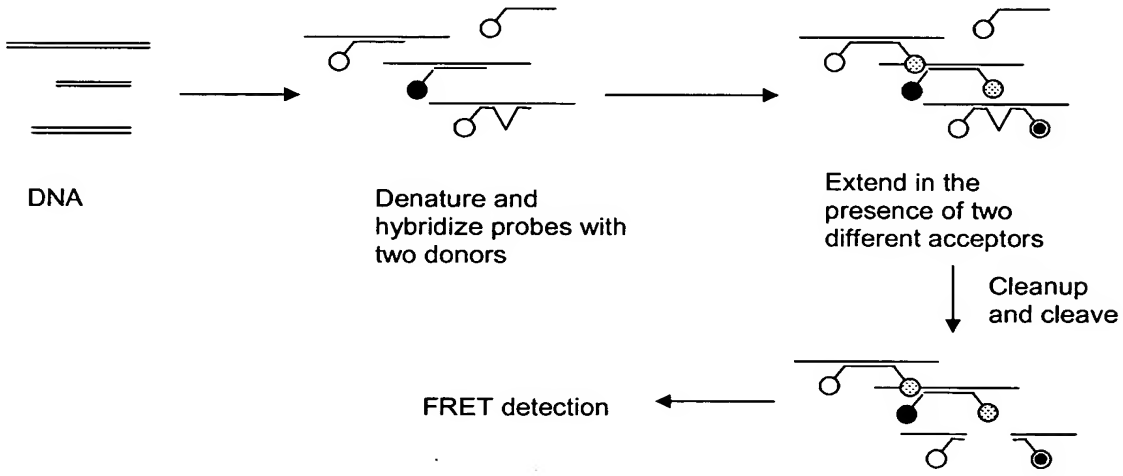

DNA
Denature and hybridize probes with two donors
Extend in the presence of two different acceptors
Cleanup and cleave
FRET detection

Fig. 55

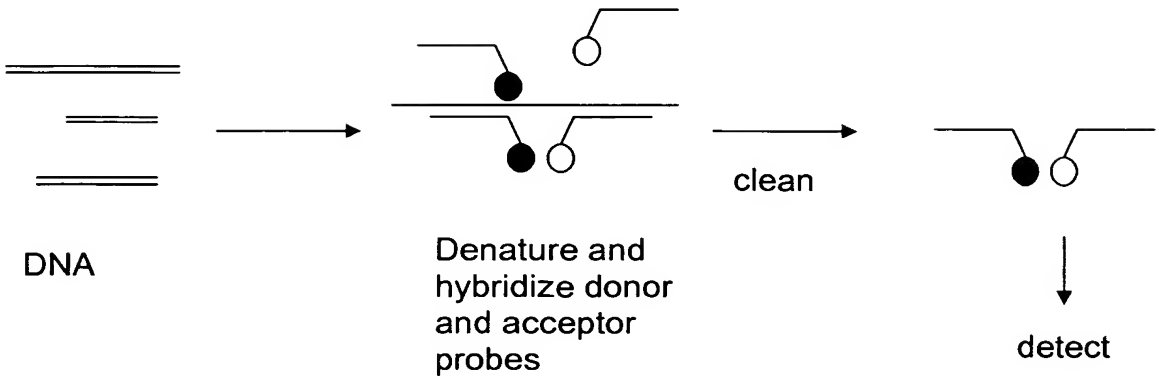

clean
DNA
Denature and
hybridize donor
and acceptor
probes
detect

Fig. 56

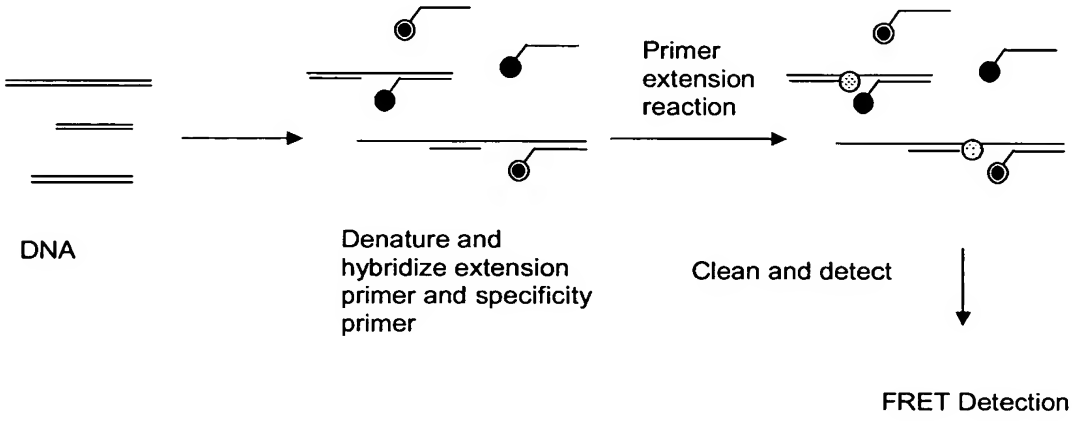
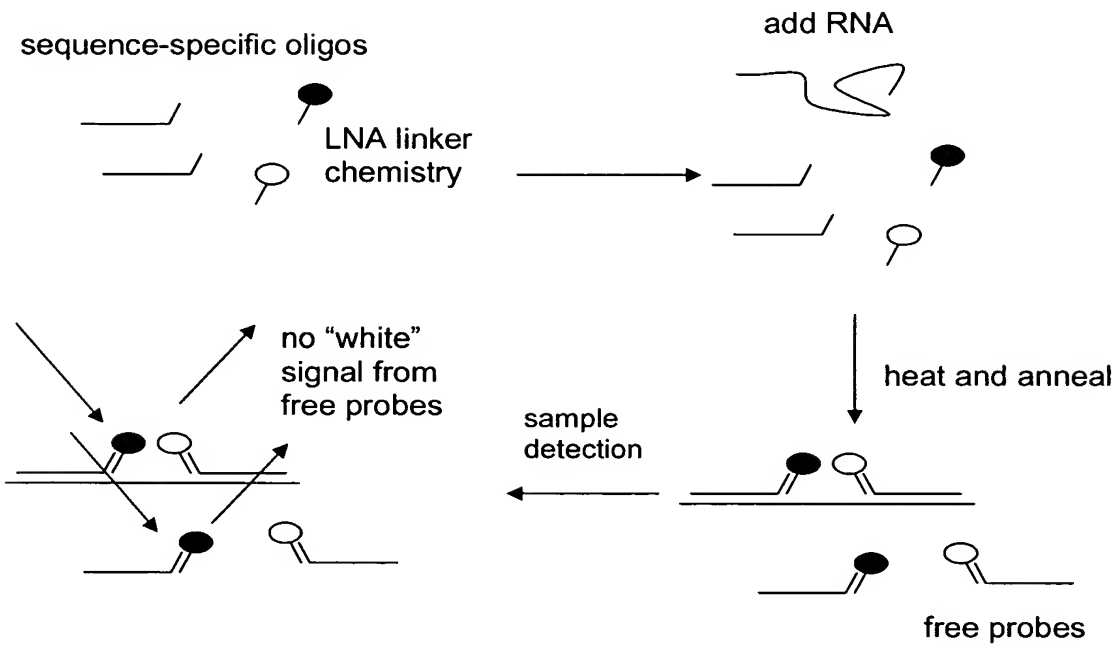

Primer
extension
reaction
DNA
Denature and
hybridize extension
primer and specificity
primer
Clean and detect
FRET Detection

Fig. 57


sequence-specific oligos
LNA linker
chemistry
add RNA
heat and anneal
free probes
sample
detection
no "white"
signal from
free probes

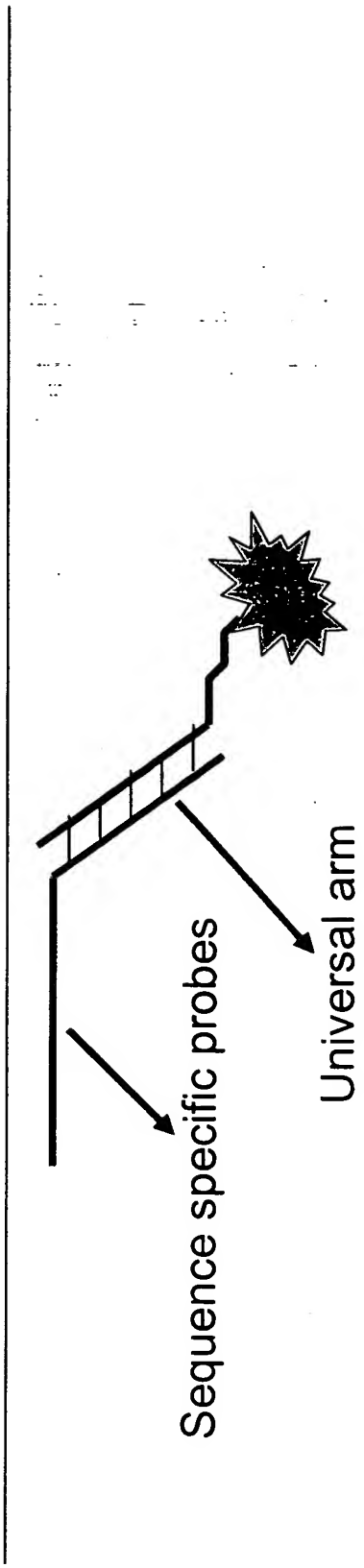


Fig. 5B